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ANDROGEN EFFECTS ON NC ENZYME AND CHROMATIN STRUCTURE IN RAT VENTRAL PROSTATE

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ANDROGEN EFFECTS ON NC ENZYME AND CHROMATIN STRUCTURE IN RAT VENTRAL PROSTATE submitted by J. DOUGLAS FILIPENKO in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry.

DEDICATION

I wish to dedicate this thesis to my parents, Norma and William Filipenko, who have always given me support and encouragment, and especially to Margot, without whom, this work would not have been possible.

ABSTRACT

The response of rat prostatic nicking-closing (NC) enzyme to withdrawal and replacement of androgens was studied to gain further understanding of the mechanism of action of steroid hormones and the *in vivo* role of NC enzyme. Also, the structure of prostatic chromatin from intact and castrated rats was compared to determine whether androgens influence chromatin structure.

An enzyme capable of relaxing negatively supercoiled DNA was detected in extracts of rat prostatic nuclei. Using a method to compare different amounts of assayable NC enzyme activity present in nuclear extracts, it was observed that the activity declined to 10% of normal values within 7 days after castration. Reduction of NC enzyme activity in castrated rats was prevented by daily injections of dihydrotestosterone. Normal NC enzyme activity levels were restored in 7-day castrated rats by 8 days of daily injections of dihydrotestosterone. This implies that assayable levels of NC enzyme activity are influenced by androgens. Return of NC enzyme activity was most rapid during the period of DNA synthesis and cell proliferation in regenerating rat prostate.

The structure of prostatic chromatin from intact and 7-day castrated rats was compared, using micrococcal nuclease as a structural probe. Electrophoretic analysis of the size of micrococcal nuclease generated DNA fragments yielded information concerning the nucleosome repeat length, nucleosome core structure and the arrangement of nucleosomes along the chromatin fibre. None of these parameters changed

in response to androgen withdrawal by castration.

It is concluded that androgens influence the activity of prostatic NC enzyme but do not appear to alter the gross structure of prostatic chromatin.

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LIST OF ABBREVIATIONS

A_{260}, A_{600}	absorbance at the specified wavelength: 260 nanometres or 600 nanometres
cm	centimetre
g	centrifugal force relative to gravity
DNA	deoxyribonucleic acid
dpm	disintergrations per minute
λem	emission wavelength
N	equivalents per litre
Na_4EDTA	ethylenediamine-tetraacetic acid, tetrasodium salt
λex	excitation wavelength
g	gram
h	hour
µg	microgram
µl	microlitre
mAmp	milliampere
mg	milligram
ml	millilitre
mm	millimetre
mM	millimoles per litre
min	minute
M	moles per litre
nm	nanometre
NC enzyme	nicking-closing enzyme, topoisomerase
Triton N-101	nonyl phenoxy polyethoxyethanol
rpm	revolutions per minute

RNA	ribonucleic acid
sec	second
S.D.	standard deviation
V	volt
vol	volume
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

A. Endocrine Control of the Prostate

The prostate gland is one of several male accessory sex tissues of reproduction which are characterized by total dependence on androgens for normal growth and function. Withdrawal of androgens by castration causes a degeneration of the prostate gland structure. The process of involution is accompanied by numerous cytological, biochemical and functional changes. Moore et al (1930) first documented the breakdown of cellular structure caused by castration. The changes in cellular structure, which are mainly confined to epithelial cells (MacKenzie et al, 1963), have been studied in detail with electron microscopy. Among the changes documented by Brandes (1974) include the collapse and degradation of the rough endoplasmic reticulum, Golgi apparatus and secretory vesicles; changes in the distribution and concentration of mitochondria; depletion of polysomes and free ribosomes; and conversion of the symmetrical nucleus into a more irregular structure. These cytological changes are associated with the appearance of a large number of lysosomes (Brandes, 1974) which are believed to be responsible for the degradation of cellular organelles (de Duve, 1959).

Biochemical changes which are induced by castration include a decline in the production of prostatic secretory products - citrate, fructose and acid phosphatase (Brandes and Bourne, 1963; Mann, 1964; Kircheim and Scott, 1965); a general reduction in RNA and protein biosynthesis (Brandes and Bourne, 1963; Williams-Ashman et al, 1964; Mangan et al, 1967); and a decline in the rate of oxygen consumption and respiration-coupled metabolic activities (Nyden and Williams-Ashman,

1953; Butler and Schade, 1958). Parker and Mainwaring (1977) have reported that there are changes in the abundance of specific poly(A)-containing RNA sequences, in response to castration. DNA synthesis, cellular proliferation and tissue weight also decline following castration (Lesser and Bruchovsky, 1973; Tuohimma and Niemi, 1974). Castration drastically reduces the concentration of nuclear androgen receptor proteins, which are believed to be involved in mediating the stimulatory and homeostatic effects of androgens (Van Doorn et al, 1976).

Regeneration of the prostate gland in castrated rats can be achieved with administration of testosterone or dihydrotestosterone at regular intervals. All the structural elements of prostate cells return with hormone replacement therapy (Moore et al, 1930; Brandes, 1974). Ribosomal RNA synthesis is stimulated very early after administration of hormones (Coffey, 1974). A lag period of 1-2 days is observed before DNA synthesis and cellular replication begins (Lesser and Bruchovsky, 1973). During this lag period there is an increase in the concentration of nuclear androgen receptors. This increase may be responsible for controlling the entry of the cell into the cell cycle (Van Doorn et al, 1976). There is also a general increase in RNA and protein synthesis during the administraiton of androgens (Liao and William-Ashman, 1962; Williams-Ashman et al, 1964; Mangan et al, 1967; Chung and Coffey, 1971; Mainwaring and Wilce, 1972). Continued administration of androgens after the prostate has attained its normal size appears to be a stimulus for secretory activity, suggesting that regeneration of the prostate may occur through a sequence of proliferation followed by differentiation (Lesser, 1974).

Much work has been done concerning the mechanism of action of

of steroid hormones (for a review see Mainwaring, 1978). Attention has focused upon specific hormone-inducible proteins and cellular functions. This thesis deals with the influence of androgens on two possible control elements of DNA synthesis and transcription; first, an enzyme capable of altering the conformation of chromatin, and second, the structure of chromatin itself.

B. NC Enzyme

DNA, like all other biological macromolecules, can possess tertiary structure in addition to the more familiar primary and secondary structures that are characteristic of this molecule. DNA tertiary structure refers to the helical winding of the DNA duplex around the duplex axis. The presence of tertiary structure demands the DNA be constrained and this condition forces the number of superhelical turns plus the number of turns of the double helix to remain a constant. Any change in the winding of the double helix must produce concommitant changes in the degree of supercoiling and vice versa. Supercoiling and coiling of the double helix are related such that any effort to increase the winding of the double helix leads to an increase in the number of negative supercoils while any decrease in the winding of the double helix induces positive supercoiling. The condition for constraint is that the free rotation of the strands of the DNA double helix is restricted at two different points. Only through DNA strand scission can the superhelical state of a constrained molecule be altered without affecting the winding of the DNA double helix.

Enzymes able to relax superhelical DNA molecules without altering their particular conditions of constraint do so by

introducing a transient nick into one of the strands of the DNA helix (Champoux, 1977). The existence of such proteins was first postulated to provide a mechanism allowing rotation of the duplex DNA ahead of a replication fork in replicating DNA molecules (Wang, 1973). Also, if the DNA molecule is constrained ahead of the replication fork, semi-conservative replication would introduce positive supercoiling in the DNA preceeding the replication fork. A mechanism to remove such superhelical twists is necessary if DNA replication is to proceed unhindered (Burrington and Morgan, 1976).

Superhelix unwinding proteins are also postulated to be necessary for DNA transcription. If an enzyme could provide a transient nick to allow the DNA template to rotate during transcription, nascent RNA molecules and attached transcribing ribosomes could remain stationary (Wang, 1973). However, NC enzyme and superhelicies have also been postulated to serve as transcriptional control elements. Superhelical twists present in covalently closed circular DNA molecules affect the frequency of RNA chain initiation by RNA polymerase. Negatively supercoiled substrates appear to serve as better templates for in vitro transcription than similar molecules without supercoils (Botchan et al, 1973; Richardson, 1974). Observations such as these suggest that NC enzyme might be able to influence rates of transcription by altering the extent of supercoiling of DNA templates (Burrington, 1977).

The activity of NC enzyme was studied in nuclei isolated from prostates of intact rats, 7-day castrated rats and 7-day castrated rats receiving androgen replacement therapy. The objective was to determine the variation of NC enzyme activity after castration and

during hormone replacement therapy. With this information and the accumulated knowledge concerning cellular proliferation and differentiation in the prostate, it was hoped that some conclusions could be made concerning the *in vivo* function of prostatic NC enzyme.

C. Chromatin Structure

This section will only draw attention to the features of chromatin structure that relate to the material presented in this thesis. For an extensive review of the literature concerning the structure of chromatin, the reader is refered to Felsenfeld (1978).

At the focal point of the currently accepted model of chromatin structure lies the nucleosome. DNA contained in chromatin is complexed with proteins to form structures known as nucleosomes (Van Holde *et al*, 1974). This DNA can be characterized by its susceptibility to micrococcal nuclease. Approximately 65% of the DNA in chromatin is nuclease resistant (intranucleosomal or core DNA) and the remainder is nuclease sensitive (internucleosomal or spacer DNA). Under the electron microscope, chromatin has a structure resembling "beads on a string". The "beads" refer to the nucleosome core which appear as particles roughly spherical in shape and the "string" refers to the lengths of spacer DNA which connect the nucleosome cores (Olins and Olins, 1974; Van Holde *et al*, 1974).

The nucleosome core contains approximately 140 base pairs of DNA coiled around a protein core consisting of two each of histones H2a, H2b, H3 and H4 (Kornberg, 1974; Van Holde *et al*, 1974). This DNA-protein complex protects the DNA from exhaustive nuclease hydrolysis. Core DNA is hydrolyzed by micrococcal nuclease at a slower rate than

spacer DNA. Also, micrococcal nuclease may recognize specific geometric patterns formed by histones and DNA in the nucleosome core, such that hydrolysis of core DNA produces a unique pattern of DNA fragments which may reflect the internal structure of the nucleosome core (Van Holde et al, 1974; Whitlock, 1977).

Nucleosome cores are connected by lengths of DNA not tightly complexed with proteins but believed to be associated with at least histone H1 (Noll and Kornberg, 1977). Because the spacer DNA is not tightly complexed with proteins, it is hydrolyzed by micrococcal nuclease at a faster rate and with less discrimination than nucleosome core DNA.

The length of DNA contained in the nucleosome core plus the average length of the spacer DNA defines the quantity refered to as the average nucleosome repeat length. Since the length of DNA contained in the nucleosome core appears to be constant regardless of the source (Compton et al, 1976), any differences in the repeat lengths observed between different organisms are derived from variations in the length of the spacer DNA (Felsenfeld, 1978).

Limited hydrolysis of chromatin by micrococcal nuclease leads to the production of DNA fragments that, when isolated and analyzed electrophoretically, are found to have lengths equal to integral multiples of a basic repeating unit. The average basic repeat length is slightly smaller than the average nucleosome repeat length and represents the length of DNA that was contained in a nucleosome core plus some of the DNA contained in the adjacent spacer regions. The average basic repeat length would equal the average nucleosome repeat length at the limit of zero percent hydrolysis. Hydrolysis at points

spanning more than one nucleosome core, produces DNA fragments whose sizes are integral multiples of the average basic repeat length. By analyzing the sizes of these micrococcal nuclease generated DNA fragments, information concerning the average nucleosome repeat length and the arrangement of nucleosome cores along the chromatin fibre can be obtained (Lohr et al, 1977; Noll and Kornberg, 1977).

Although much has been learned about the structure of chromatin, little is known how the structure of chromatin relates to its function as a template for DNA synthesis and transcription. In this thesis, micrococcal nuclease was used to probe the nucleosome structure of prostatic chromatin from intact and 7-day castrated rats. The purpose was to determine if the structure of prostatic chromatin in intact rats changed when the gland was deprived of androgens.

CHAPTER II

MATERIALS AND METHODS

A. Sources

Experimental animals were Wistar rats, obtained from Woodlyn Laboratories, Guelph, Ont.

PM2 DNA was either obtained as a gift from Dr. A.R. Morgan, University of Alberta, or purchased from Boehringer Mannheim GmbH Biochemica, Mannheim, W. Germany.

Micrococcal nuclease E.C.3.1.4.7. and trypsin were purchased from Sigma Chemical Co., St. Louis, Mo. Hae III restriction endonuclease was purchased from Bethesda Research Laboratories, Inc., Rockville, Md.

Electrophoretic grade agarose, acrylamide and ethidium bromide were supplied by Sigma. N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide (bis) and Stains-all were purchased from Eastman Kodac Co., Rochester, N.Y.

Steroids were products of Steraloids, Pawling, N.Y.

Anaesthetic grade diethylether was purchased from Mallinckrodt, Inc., St. Louis, Mo.

All other chemicals used to prepare buffers were at least reagent grade. Deionized glass distilled water was used to prepare all solutions.

B. Buffers

Buffer A : 10 mM-Tris, 5 mM-$MgCl_2$, 0.5 mM-β-mercaptoethanol, pH 8.

Buffer B : 1 mM-Tris, 1 mM-$CaCl_2$, pH 8 containing 0.44 M-sucrose and 0.3% (v/v) Triton N-101.

Buffer C : 1 mM-Tris, 1 mM-$CaCl_2$, pH 8 containing 0.25 M-sucrose.

Buffer D : 10 mM-Tris, 1 mM-Na_4EDTA, 0.2 M-NaCl, pH 8 containing 1-2 A_{260} units of PM2 DNA.

Buffer E : 5 M-NaCl : 10 mM-Tris, 1 mM-Na_4EDTA, pH 8 : 40% (w/v)polyethylene glycol 6000 (2:2:1 by vol).

Buffer F : 20 mM-KH_2PO_4, 0.1 mM-Na_4EDTA, 0.5 µg/ml ethidium bromide, pH 11.6.

Buffer G : 40 mM-Tris, 20 mM-Na_4EDTA, 20 mM-$NaCH_3COO$, pH 7.8.

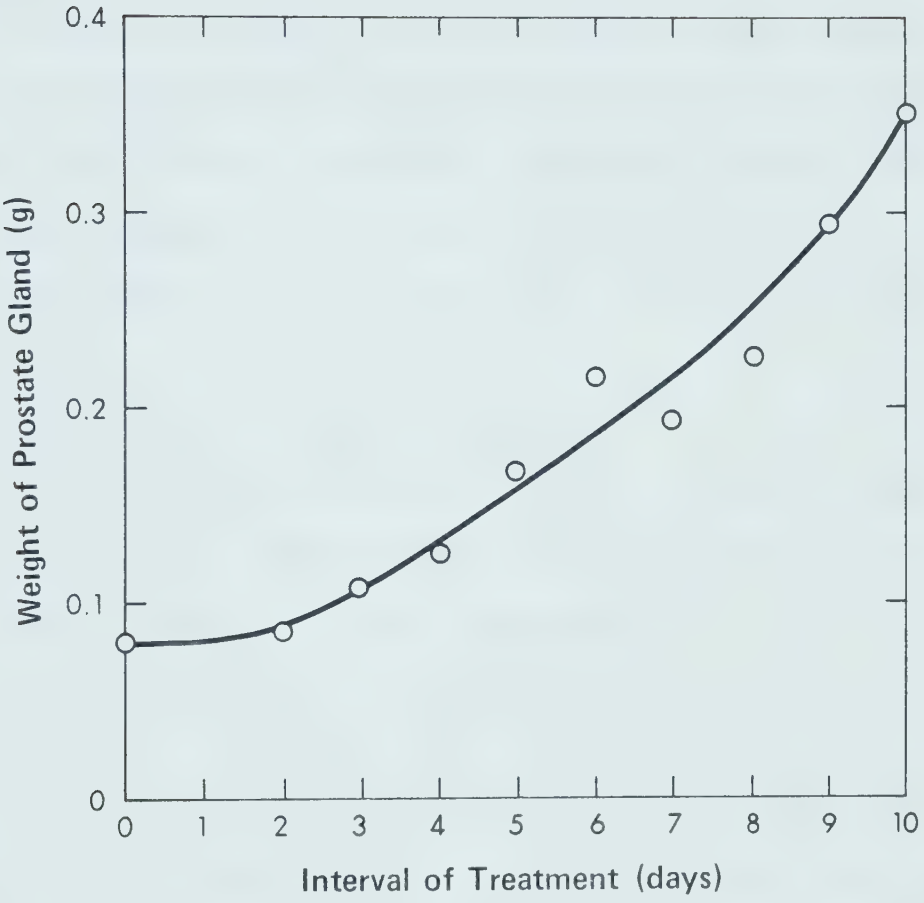
C. Animals

Adult, male Wistar rats (250-350 g) received a diet of Purina Rat Chow and water, ad libitum. Rats were castrated via the scrotal route while anaesthetized with ether. All rats were killed by decapitation.

Rats receiving androgens were given daily subcutaneous injections of 400 µg steroid per 100 g body weight. Ether was not used to subdue the animals prior to injection. The steroid dose was administered in 1.0 ml of 10% (v/v) ethanol and 10% (v/v) polyoxyethylene sorbitan monopalmitate (Tween 40).

Prostrate ventral lobes were immediately dissected from decapitated rats, stripped of external connective tissue and placed in a beaker. At least 1 g of tissue was necessary for an NC enzyme assay and 2 g for a micrococcal nuclease digestion experiment. One g of tissue was the amount recovered from 4 intact rats or 18 7-day castrated animals. The weight of prostates from 7-day castrated rats receiving exogenous dihydrotestosterone increased as a function of treatment time (Figure 1). Hence, as treatment with dihydrotestosterone continued, fewer animals

Figure 1 Variation of prostatic tissue weight in 7-day castrated rats receiving dihydrotestosterone. Groups of 4-15 castrated rats received daily subcutaneous injections of dihydrotestosterone (400 μg per 100 g body weight) commencing 7 days after castration. Ventral lobes of the prostate gland were removed at various times after initiation of treatment, stripped of connective tissue and weighed. Results are expressed as the mean weight of pooled prostates from two separate determinations. The ventral lobes of the prostate from intact rats (250-350 g) weighed an average of 0.37 g.


0.4
0.3
0.2
0.1
0
Weight of Prostate Gland (g)
0
1
2
3
4
5
6
7
8
9
10
Interval of Treatment (days)

were needed to obtain the requisite amount of tissue. By 10 days of androgen replacement therapy, prostatic weight approached that of tissue found in intact rats. This observation is in agreement with published results (Lesser and Bruchovsky, 1973; Tuohimaa and Niemi, 1974).

D. Isolation of Prostatic Nuclei

Prostatic nuclei were isolated by different methods depending upon the type of experiment. Nuclei to be used for NC enzyme activity assays had to be extensively purified to minimize the level of cytoplasmic contamination. However, chromatin structure analysis demanded a less rigorous purification procedure so that more nuclei could be isolated per g of tissue.

1. High purity, low yield procedure

All operations were carried out at 0-4^{o}C. Freshly dissected prostatic tissue (1-2 g) was thoroughly minced with scissors and forced through a 30 gauge stainless steel screen with the aid of a teflon-tipped aluminium pestle. The minced tissue was washed through the screen with 25-30 ml of buffer A containing 0.25 M-sucrose and 1.5 mM-$CaCl_2$. The resulting suspension was then homogenized in a ball-type Dounce homogenizer with 25 strokes of the loose fitting pestle. At this point the suspension was filtered through 2 layers of cheese cloth to facillitate the second homogenization of 20 strokes with the tight fitting Dounce pestle. This homogenate was centrifuged at 800g for 10 min (all samples centrifuged in a Sorval RC-5 centrifuge, DuPont Company, Newtown, Conn., unless otherwise noted). The nuclear pellet was resuspended in 25 ml of buffer A containing 0.88 M-sucrose and 1.5 mM-$CaCl_2$ with 20 strokes of the tight fitting pestle. This

suspension was then divided into portions containing the equivalent of 0.5 g tissue. Each portion was then made up to 25 ml with buffer A containing 0.88 M-sucrose and 1.5 mM-$CaCl_2$ and used to prepare a discontinuous sucrose gradient together with 5 ml of buffer A containing 1.8 M-sucrose and 1.5 mM-$CaCl_2$ on a cushion of 5 ml of buffer A containing 2.2 M-sucrose and 1.5 mM-$CaCl_2$. These gradients were centrifuged at 53,000g for 90 min (Beckman L2-65B ultracentrifuge, Beckman Instruments, Inc., Palo Alto, Ca.; SW 27 rotor).

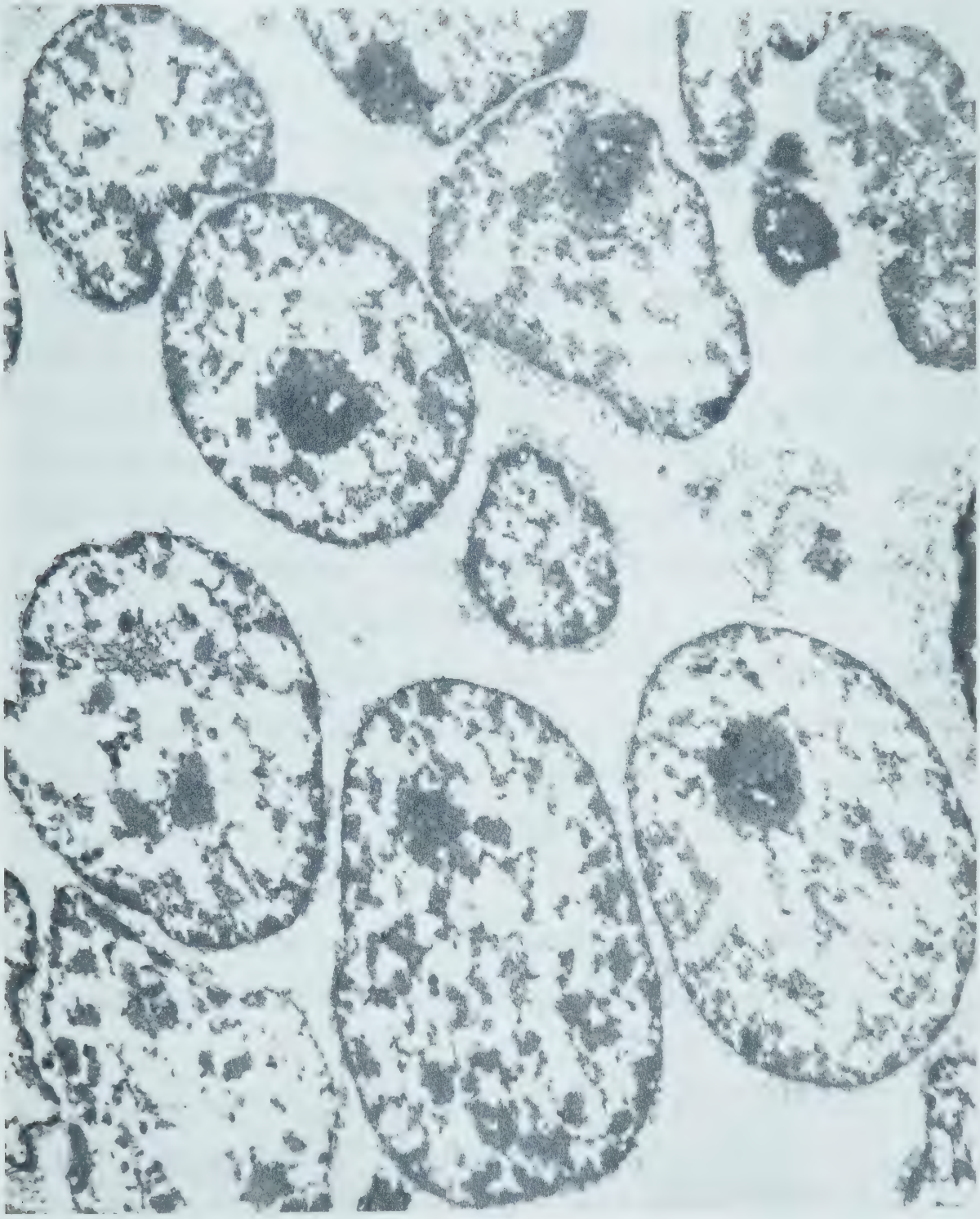
After the spin, an aspirator was used to remove the supernatant. The pellet was resuspended in 2.0 ml of buffer A containing 50 mM-NaCl and 33% (v/v) glycerol by gently passing the contents through a 20 gauge needle attached to a 3 ml syringe.

Nuclei yields were estimated by diluting 100 µl of this final nuclear suspension in 2.0 ml buffer A containing 50 mM-NaCl, 33% (v/v) glycerol and 0.05% (w/v) methylene blue. The nuclei were counted using a Spencer Bright-Line hematocytometer (American Optical Corp., Buffalo, N.Y.). Typical nuclear yields ranged between $1.7X10^7$ and $4.6X10^7$ nuclei per g of prostatic tissue, with a mean of $3.2X10^7$ nuclei per g.

Nuclear suspension thus purified contained little debris as seen under the light microscope. Electron micrographs of prostatic nuclei purified in the same manner revealed intact nuclear membranes with little or no adhering cytoplasm (Figure 2).

Nuclei were immediately used to prepare nuclear extracts for assaying NC enzyme activity. Nuclei could be stored as a suspension in buffer A containing 50 mM-NaCl and 33% (v/v) glycerol for up to 6 months at -40^oC. Little damage to the gross structure of the

Figure 2 Electron micrograph of intact rat prostatic nuclei prepared using the high purity, low yield procedure described in Chapter II.D.1. Magnification is approximately 10,000x.



nuclei was noted. However, as little as 1-2 weeks of storage at -40°C resulted in substantial reduction of NC enzyme activity. Thus, all nuclei were assayed for NC enzyme activity as soon as they were purified.

2. Low purity, high yield procedure

The procedure followed was as outlined in Chapter II.D.1. up to the recovery of the 800g nuclear pellet. At this point the pellet was suspended in 25 ml of buffer B using 15 strokes of the tight fitting Dounce pestle. This suspension was centrifuged at 400g for 15 min. The pellet was washed with 10 ml buffer C, using 10 strokes of the tight fitting Dounce pestle. The suspension was centrifuged at 600g for 15 min and the pellet resuspended in buffer C (1-2 ml per g of prostatic tissue) by gently passing the contents through a 20 gauge needle attached to a 3 ml syringe. This suspension of nuclei was immediately used for investigating chromatin structure.

Yields of nuclei from both intact rat prostate (range, $5.1X10^7$ - $7.7X10^7$ nuclei per g of prostate ; mean, $6.6X10^7$ nuclei per g) and 7-day castrated rat prostate (range, $4.0X10^7$ - $7.1X10^7$ nuclei per g of prostate ; mean, $5.7X10^7$ nuclei per g) were clearly higher using this method to isolate nuclei.

Isolated nuclei were free from adhering cytoplasm but there was contaminating cellular debris present in the nuclear suspension.

E. NC Enzyme Reaction Conditions

1. Preparation of nuclear extract

Freshly isolated nuclei ($1-2X10^7$ nuclei in 1 ml) were centrifuged at 6000g for 2 min in an Eppendorf microfuge (Brinkman Instruments, Rexdale, Ont.) and the supernatant was carefully removed. Greater than

98% of the nuclei were recovered in the pellet. The pellet was dispersed in 100 µl of 5M-NaCl with the aid of a vortex mixing apparatus and allowed to sit on ice for 3 min. Then, 100 µl of buffer (10 mM-Tris, 1 mM-Na_4EDTA, pH8) and 50 µl of 40% (w/v) polyethylene glycol 6000 were added. After standing a further 3 min the mixture was centrifuged at 12,000g (Eppendorf microfuge) for 3 min. After centrifugation about 50% of the supernatant, which contained the NC enzyme activity, was withdrawn and assayed immediately.

2. Preparation of cytosol fraction

Intact rat prostatic tissue was treated as described in Chapter II.D.1. The 800g supernatant was saved and further centrifuged at 7800g for 20 min. The supernatant was then centrifuged at 105,000g for 60 min (Beckman ultracentrifuge; SW41 rotor). Five ml of this final supernatant was concentrated 50-fold in an Amicon Minicon-B15 macrosolute concentrator (Amicon Corporation, Lexington, Mass.). This concentrate was also assayed for NC enzyme activity. However, no NC enzyme activity could be detected and further experiments on the cytosol fraction were not performed.

3. Fluorescence assay of NC enzyme activity

The procedure developed by Morgan and Pulleyblank (1974) was used to measure NC enzyme activity.

NC enzyme activity was measured at room temperature (23°C) by pipetting 30 µl of intact rat prostatic nuclear extract into 600 µl of buffer D. The control sample (zero time point) consisted of 5 µl of buffer E per 100 µl of buffer D. At various times after addition of nuclear extract, four 20 µl samples were removed and each placed into 2.0 ml of buffer F. Four 20 µl samples were also removed from the

control and each added to 2.0 ml of buffer F.

All samples and blank (buffer F) were then heated at 100°C for 2 min and rapidly cooled to room temperature. An Aminco-Bowman spectrofluorimeter (American Instruments Co., Silver Spring, Md.) was used to measure the fluorescence of samples and blank (λ_{ex}526 nm, λ_{em}600 nm). Fluorescence intensity measurements were recorded by a Brinkman 2543 strip chart recorder (Brinkman Instruments, Westbury, N.Y.).

All results are expressed as percent decrease in fluorescence enhancement (FE), $[(FE_{control}-FE_{sample}) \div (FE_{control})] \times 100\%$, where FE is the fluorescence of the sample less the fluorescence of the blank.

To quantitate the amount of assayable NC enzyme activity in any given extract, the extract was serially diluted with buffer E. Each dilution was then assayed by pipetting 5 µl into 100 µl of buffer D. After a 60 min incubation at room temperature, four 20 µl samples were removed and each placed into 2.0 ml of buffer F. A control was prepared as described above. All samples were heated, cooled and their fluorescence values recorded.

4. Trypsin sensitivity of NC enzyme activity

A solution of trypsin (5 mg/ml in buffer E) was freshly prepared. An extract of intact rat prostatic nuclei was prepared as described. The extract was diluted 2-fold with both buffer E and the trypsin solution. Both diluted solutions and the original extract were incubated at room temperature for 30 min prior to being assayed for NC enzyme activity.

The extract diluted with trypsin solution showed no NC enzyme activity, while both the original extract and the extract diluted with

buffer E fully relaxed the PM2 DNA substrate under the conditions of the fluorescence assay. This implies that the agent responsible for relaxing PM2 DNA is a protein component of the nucleus.

F. Agarose Gel Electrophoresis of PM2 DNA

To confirm that the fluorescence assay was actually measuring an enzyme capable of relaxing negatively supercoiled DNA, agarose gel electrophoresis was used to demonstrate the relaxation process. Cylindrical agarose gels (1.2% w/v) were prepared by heating sufficient agarose in buffer G to reflux, then cooling the mixture to about 60°C and pouring this solution into pre-heated 110 x 5 mm (i.d.) tubes. A small amount of hot water (60°C) was layered on top of the freshly poured gel, providing a suitable surface on which the sample was applied. The tubes were fire-polished at the lower end to provide a constriction which prevented extrusion of the gel during electrophoresis. The constricted end was also covered with a piece of Spectrapor 1 dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, Ca.), held in place by a rubber band.

The gels were loaded with 1-2 µg PM2 DNA in a volume of 25 µl. Samples were prepared by repeated freezing (liquid nitrogen) and thawing of a 100 µl reaction mixture (Chapter II.E.3.) which was subsequently adjusted to 10% glycerol. The samples were electrophoresed at a constant current of 2 mAmp for about 16 h. The disc electrophoresis apparatus (Model 1200, Canalco, Rockville, Md.) was kept at temperature of 23-28°C by water circulating through a cooling jacket.

Gels were stained with ethidium bromide (10 µg/ml) for 5-10 min. DNA showed up as orange bands under the illumination of a short wave ultraviolet light. Photographs were taken with a camera

equipped with a 35 mm lens and an orange filter, under the direct illumination of short wave ultraviolet light.

G. Micrococcal Nuclease Digestion of Prostatic Nuclei

The structure of rat prostatic chromatin was studied using micrococcal nuclease to partially hydrolyze chromatin contained in whole nuclei. To freshly isolated nuclei (Chapter II.D.2) was added a small volume (0.6 ml) of buffer C containing micrococcal nuclease (final enzyme and DNA concentrations were 10 units per ml and 100-200 μg of DNA per ml). All digestions were carried out at room temperature. Digestion times were varied to liberate different amounts of acid soluble nucleotides. The reaction was stopped by bringing the final EDTA concentration to 20 mM with 0.5 M-Na_4EDTA, pH 7.7. A small volume of nuclear suspension was similarly treated with buffer C (no added nuclease) and EDTA to serve as a control.

H. Analysis of Digestion Products

DNA concentration of nuclear suspensions were measured by placing 25 μl of the suspension into 1.0 ml 1N-NaOH, heating for 3 min at 100°C in a Tecam dri-block (Techne Inc., Princeton, N.J.) quickly cooling to room temperature and measuring A_{260} against 1N-NaOH in a Zeiss spectrophotometer. The percent digestion was measured by first pipetting in triplicate, 100 μl aliquots from the digested and control samples into 100 μl of 0.8 M-NaCl, 0.8 N-$HClO_4$. This mixture was placed on ice for 15 min, then centrifuged at 12,000g for 3 min in an Eppendorf microfuge. A 100 μl portion of each supernatant was added to 1.0 ml 1N-NaOH and the A_{260} was measured against 1N-NaOH. The absorbance

difference between sample and control values (corrected for dilutions) yielded a quantity proportional to the acid soluble DNA concentration present in the digested sample. Percent digestion was calculated upon dividing the amount of acid soluble DNA by the total amount of DNA present in the sample.

It was found that 1 A_{260} unit (measured in 1N-NaOH) was equivalent to 30 µg DNA per ml. Using this relation it was calculated that the DNA content of prostatic nuclei was 119 $\pm$ 40 µg DNA per 10^7 nuclei (mean $\pm$ S.D., n=5) for intact rat prostate and 121 $\pm$ 38 µg DNA per 10^7 nuclei (mean $\pm$ S.D., n=6) for 7-day castrated rat prostate.

The percent digestion as a function of the micrococcal nuclease concentration, DNA concentration and the time of digestion, are shown in Figure 3. If both DNA and nuclease concentrations are known, the relationship enables one to determine the digestion time required to produce a desired range of percent digestion.

I. Isolation of DNA from Prostatic Nuclei

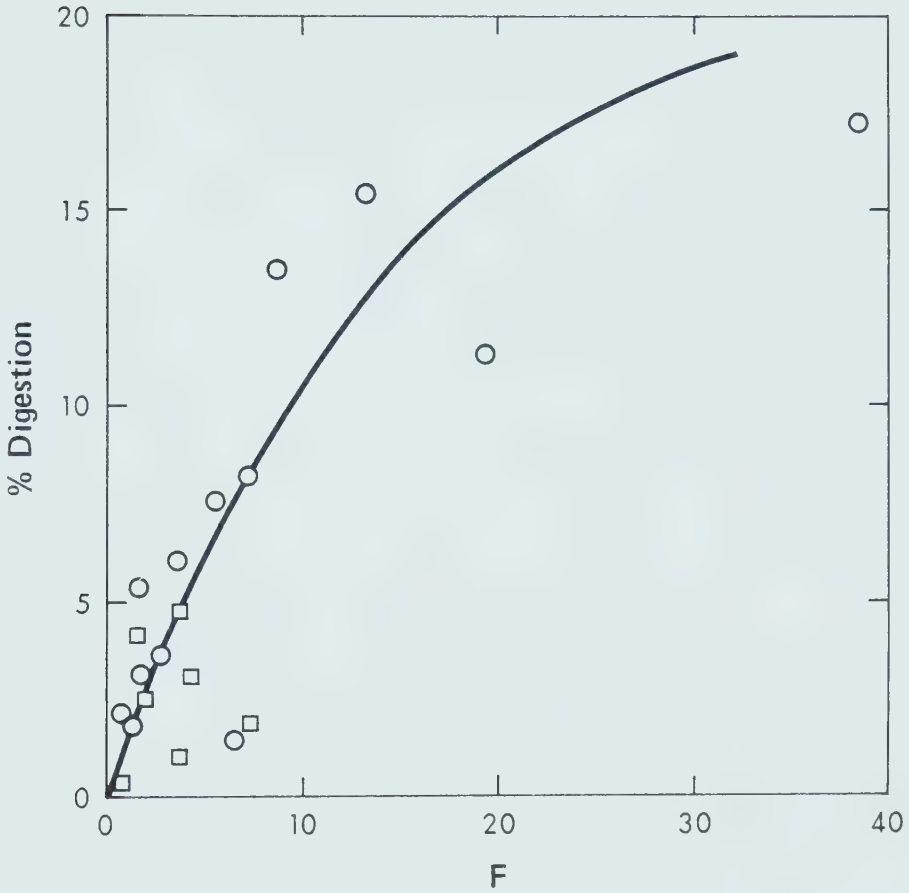
The intact rat prostatic nuclei digestion experiments yielded DNA concentrations sufficiently high enough to isolate the DNA fragments by a modified Marmur technique (Britten et al, 1974).

Digested nuclei suspensions (1-2 ml) were mixed with 0.1 vol 5M-$NaClO_4$, followed by 0.5 vol choloroform : isoamylalcohol (24:1 v/v) and 0.5 vol phenol. This mixture was shaken for 20 min on a rotating platform (Laboratory Rotator - Model G2, New Brunswick Scientific Co., New Brunswick, N.J.) at 250 rpm. The milky white emulsion was broken by centrifugation at 2700g for 5 min. This separated the DNA containing aqueous phase from the more dense organic phase. Proteins appeared as

Figure 3 Relationship between incubation time, nuclease concentration, DNA concentration and % digestion. Prostatic nuclei were isolated and incubated with micrococcal nuclease. Reactions were stopped by raising the EDTA concentration to 20 mM. Percent digestion, as determined in Chapter II.H., is plotted against a parameter, F, such that

$$F = [MN] \times T \div [DNA],$$

where [MN] is the final micrococcal nuclease concentration in enzyme units per ml, [DNA] is the final DNA concentration in µg per ml and T refers to the duration of incubation in seconds. Results include data from digestions of intact rat prostatic nuclei (○) and 7-day castrated rat prostatic nuclei (□).


20
15
10
5
0
% Digestion
0
10
20
30
40
F

a white precipitate between the two phases. The aqueous solution was transfered to a 15 ml centrifuge tube and 2 vol of cold ($0^{o}C$) absolute ethanol were carefully layered on top of the solution. The tube plus contents were left overnight at $4^{o}C$.

DNA precipitates were recovered by a 20 min centrifugation at 2700g. The pellet was washed with cold ethanol, then placed in a vaccum dessicator overnight. Pellets were dissolved in 300 µl of 10% (v/v) buffer G containing 10% (v/v) glycerol and diluted to a DNA concentration of 0.3 µg per ml.

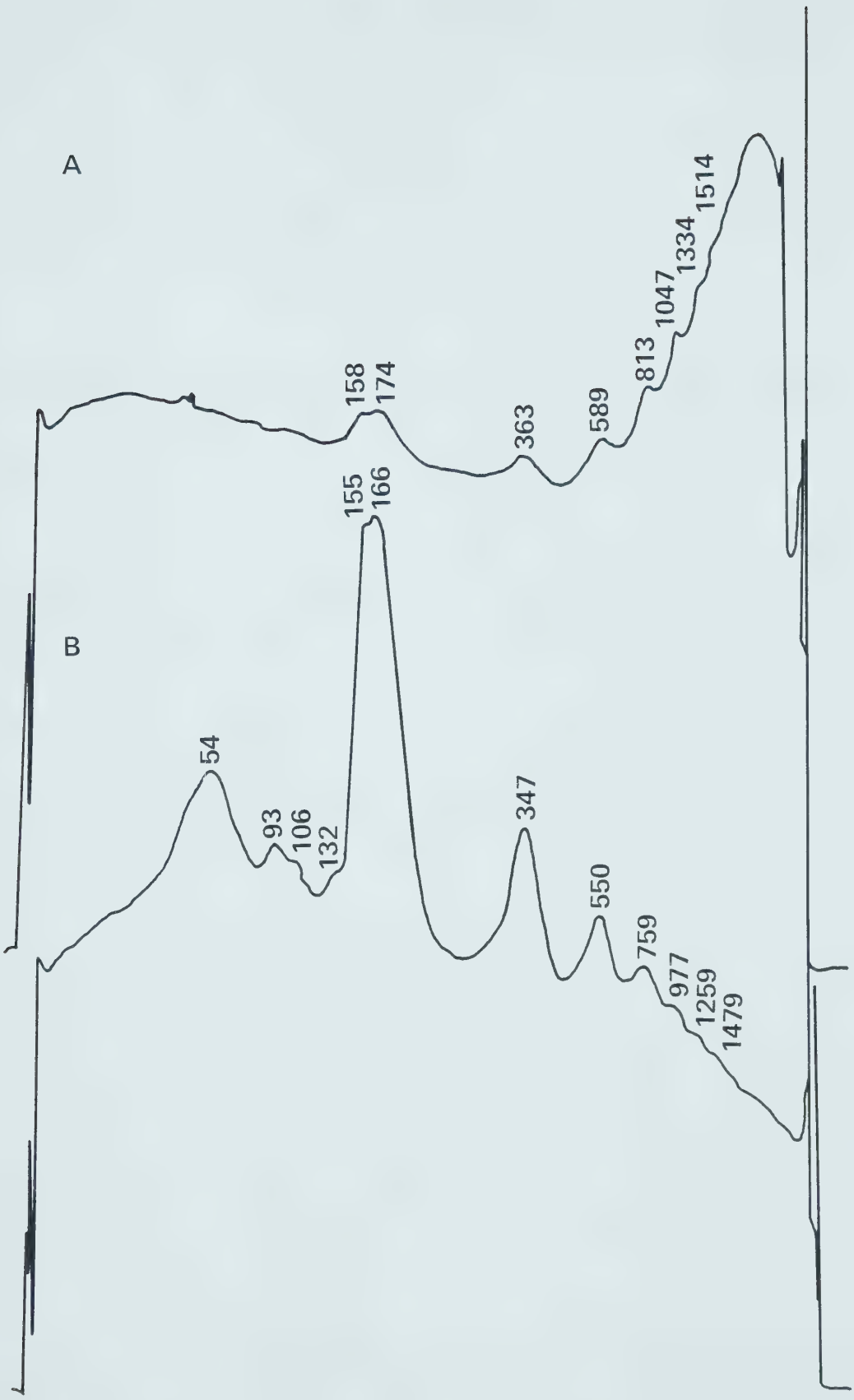
Digested prostatic nuclei from 7-day castrated rats were similarly deproteinized but ethanol precipitation did not yield satisfactory results. The ethanol precipitation was replaced by dialysis (Spectrapor 1) against 10% (v/v) buffer G. The final dialysates were concentrated in an Amicon Minicon-B15 macrosolute concentrator if the DNA concentration was less than 0.3 µg per ml. Samples were adjusted to 10% (v/v) glycerol and 0.3 µg DNA per ml.

Ethanol precipitation and dialysis isolate two different molecular size DNA fractions from the digested nuclei. As Figure 4 illustrates, ethanol precipitation isolates mainly the large molecular size fraction (1000+ base pairs) of DNA fragments. Dialysis of the solution after the ethanol treatment reveals a large quantity of intermediate (500-1000 base pairs) and low (< 300 base pairs) molecular size species including DNA fragments not observed in the ethanol precipitated fraction.

J. Preparation of Hae III Restriction Endonuclease Fragments of PM2 DNA

PM2 DNA (10 A_{260} units) in 50 mM-Tris, 5 mM-$MgCl_2$, 1 mM- β -mercaptoethanol, pH 7.5 was incubated with Hae III restriction

Figure 4 Prostatic nuclei were isolated from 7-day castrated rats and incubated with micrococcal nuclease (1.8% digestion). The chromatin contained in the nuclei was deproteinized using the technique of Britten et al (1974). The DNA was precipitated with ethanol and resuspended in 10% buffer G containing 10% glycerol, to a DNA concentration of 0.3 µg per µl (Fraction A). The unprecipitated DNA left in solution, was dialyzed against 10% buffer G, concentrated with an Amicon Minicon-B15 macrosolute concentrator and made up to 10% glycerol and 0.3 µg DNA per µl (Fraction B). Both fractions were electrophoresed on 3% acrylamide : 0.5% agarose composite slab gels as described in Chapter II.K. The gel was stained with Stains-all and scanned at 600 nm using a spectrophotometer equipped with a gel scanning accessory. Direction of migration is from right to left. DNA fragments are identified by their length in base pairs.


A
158
174
363
589
813
1047
1334
1514
B
54
93
106
132
155
166
347
550
759
977
1259
1479

endonuclease (10 units per 100 µl DNA solution) for 2 hours at 37°C. The reaction mixture was made up to 10 mM-EDTA with 0.5M-Na_4EDTA, pH 7.7 and stored at 4°C until required.

These Hae III fragments of PM2 DNA have been characterized by Kovacic and Van Holde (1977). One well in each slab gel was reserved for these fragments to calibrate the gel.

K. Size Analysis of Micrococcal Nuclease Generated DNA Fragments

DNA fragments isolated from digested prostatic nuclei were characterized on 3% acrylamide : 0.5% agarose composite gels (14 x 17 x 0.15 cm, 10 well slabs). Gels were prepared by first heating an assembled gel core (BioRad model 220 vertical electrophoresis cell) with hot tap water flowing through the cooling jacket of the apparatus.

A 1% (w/v) agarose solution in buffer G was prepared by heating to reflux. A 50 ml portion of the hot solution was immediately mixed with 50 ml 6% (w/v) acrylamide (acrylamide:bis 19:1) in buffer G, followed by 0.4 ml 10% (w/v) ammonium persulfate and 25 µl TEMED. Half the mixture was taken up in a 50 ml syringe which was then fitted with an 18 gauge needle. The solution was carefully introduced between the warmed gel forming plates attached to the cell core. A teflon well forming comb was placed between the plates to a depth of approximately 2 cm. The circulating water was changed from hot to cold and the gel allowed to set for about 1 h, before water was added to the top reservoir to aid in removing the teflon comb. The gels were prepared 1 day prior to use.

DNA samples contained glycerol (10% v/v) and from 5 to 10 µg DNA in a total vol of 30 µl. Gels were run for 3.5 h at 60 V (50 - 55 mAmp), with circulating 15°C tap water as a coolant.

Gels were stained with 0.025% (w/v) Stains-all in 50% (v/v) formamide for about 2 h and destained in water for 1 h. Gels were then cut up and scanned at 600 nm using a Gilford spectrophotometer equipped with a gel scanning accessory. The A_{600} profile of the gel was recorded using a Brinkman 2543 strip chart recorder. All gels were run, stained and scanned on the same day to minimize the effect of diffusion on band size. Distances were measured on the recorder paper using Vernier calipers.

The reproducibility of DNA fragment migration measurements was estimated. Two slab gels were prepared and run simultaneously. The migration distances of PM2 DNA Hae III fragments were measured and compared (Table 1). Differences in measured distances were greatest at the two size extremes, but never exceeded 1 mm (representing a maximum uncertainty of between 3 base pairs and 130 base pairs, depending upon the actual size of the DNA fragment in question). The greatest source of error appeared to be the procedure of cutting the slab gel into slices in preparation for analysis on the gel scanner. DNA samples tend to migrate slower at the edges of the well giving the band a U-shaped appearance. If each well is not cut so that approximately the same cross-section of each gel slice is presented to the gel scanning apparatus, then large differences in the migration of identical samples are observed. This source of error makes it necessary to analyze each DNA sample a number of times to get a reliable estimate of the fragment sizes.

The effect of DNA concentration on the migration distance was also estimated. Varying amounts of DNA fragments in identical sample volumes were analyzed electrophoretically and their absorbance profiles

Table 1 The reproducibility of measuring the migration distance of DNA fragments. Each of two samples of Hae restriction endonuclease fragments of PM2 DNA was applied to a separate 3% acrylamide : 0.5% agarose composite slab gel. Both slab gels were cast and run together as described in Chapter II.K. Each sample contained 9 μg of DNA in a 30 μl vol. Gels were stained with Stains-all and scanned at 600 nm using a spectrophotometer equipped with a gel scanning accessory. Lengths of Hae III restriction endonuclease fragments of PM2 DNA are taken from Kovacic and Van Holde (1977).

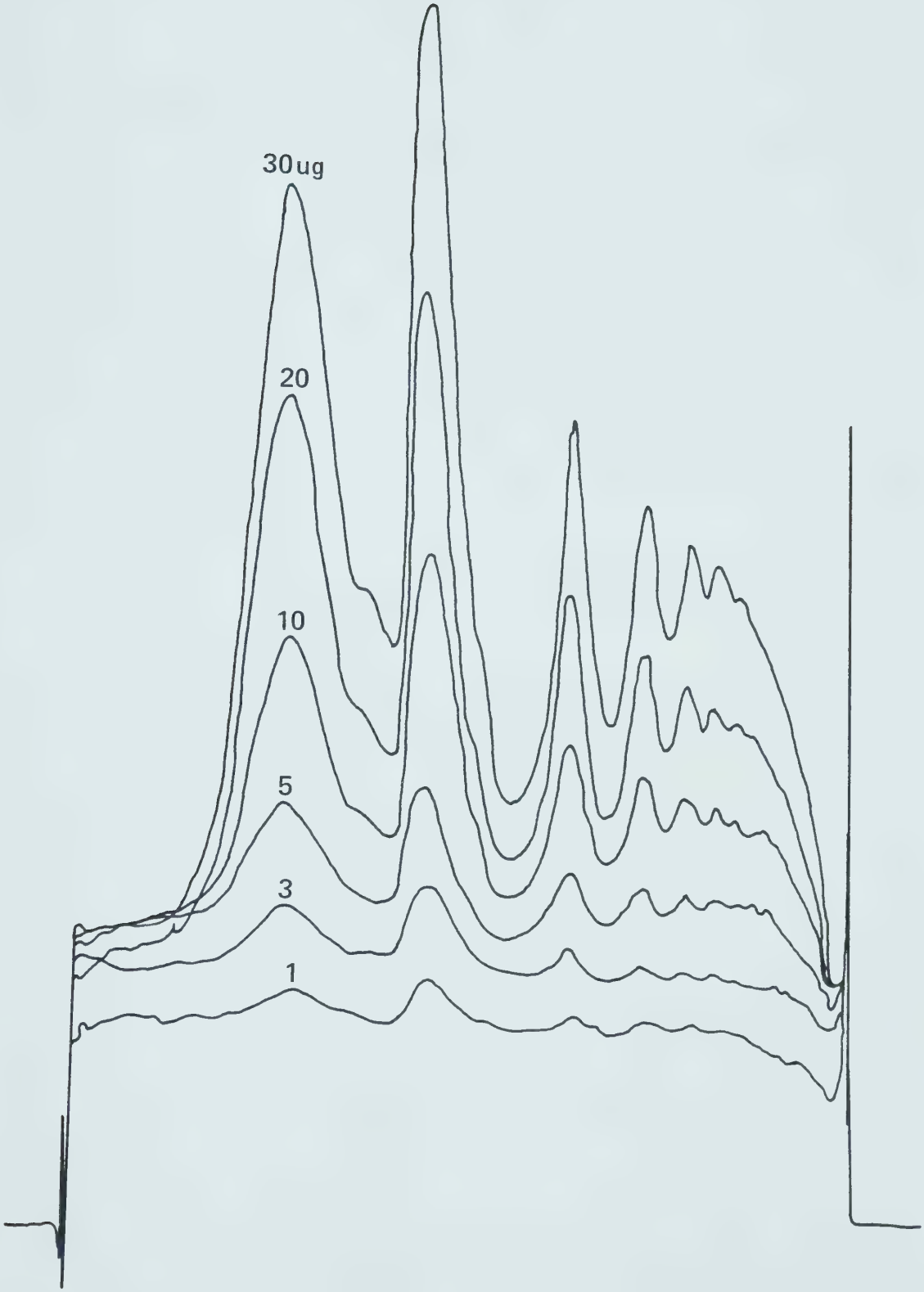
TABLE 1.

Measurement of the Migration Distances of DNA Fragments on 3% Acrylamide:0.5% Agar Composite Gels

Size of PM2 DNA Hae III fragments (base pairs)	Migration distance (mm)		
	Gel #1	Gel #2	Difference (2-1)
1860	14.20	14.75	+ 0.55
1760	16.35	16.70	+ 0.35
1410	19.30	19.45	+ 0.15
890	24.80	24.80	nil
845	27.50	27.50	nil
672	31.50	31.35	- 0.15
615	33.55	33.65	+ 0.10
525	37.50	37.75	+ 0.25
333	49.30	49.70	+ 0.40
295	52.70	53.30	+ 0.40
272	55.20	55.90	+ 0.70
167	70.25	70.95	+ 0.70
152	72.30	73.20	+ 0.90
120	78.90	79.70	+ 0.80
95	84.40	85.60	+ 0.80

compared (Figure 5). It appears that the amount of DNA loaded on the gel (at constant sample volume) does not affect the measured migration distance. A similar result is obtained if the same amount of DNA is loaded on the gel using different volumes. PM2 DNA Hae III fragments, on the average, migrated 0.6 mm slower as the sample volume increased from 15 µl through to 60 µl (at constant amount of 10 µg of DNA). However considering the accuracy with which one can expect to measure migration distances (Table 1), this difference is probably not significant.

Figure 5 Variation of the measured migration distance with the amount of DNA loaded on a slab gel. Prostatic nuclei were isolated from intact rats and incubated with micrococcal nuclease (8.2% digestion). The chromatin was deproteinized (Britten et al, 1974) and the DNA precipitated with ethanol. The unprecipitated DNA fraction was dialyzed against 10% buffer G, concentrated with an Amicon Minicon-B15 macrosolute concentrator and made up to 10% glycerol. This sample was diluted to various concentrations. A 30 µl vol of each concentration was added to one of six adjacent wells on a 3% acryalmide : 0.5% agarose composite slab gel. Gel preparation, running conditions and gel scanning procedure as described in Chapter II.K. Absorbance profiles obtained for each sample are arranged in order of increasing amount of DNA loaded on the gel. Direction of migration is from right to left. Shown in order of decreasing mobility are the fast running DNA fragments, monomers, dimers, trimers, tetramers and pentamers (Chapter III.B.1.).


30ug
20
10
5
3
1

CHAPTER III

RESULTS

A. NC Enzyme Activity

The purpose of these experiments was to demonstrate the presence of an enzyme (NC enzyme) in extracts of rat prostatic nuclei, able to relax superhelical DNA. First, a method for comparing the levels of assayable NC enzyme activity in any given nuclear extract to those present in an extract prepared from intact rat prostatic nuclei was developed. Second, the effect of castration and hormone replacement therapy on assayable NC enzyme activity, in extracts of rat prostatic nuclei, was studied.

1. Kinetics of superhelical DNA relaxation by extracts of prostatic nuclei

The fluorescence assay of NC enzyme is based upon a decrease in fluorescence enhancement reflecting the removal of superhelical turns present in the PM2 DNA substrate. PM2 DNA is a negatively supercoiled covalently closed circular DNA and as such, will bind more ethidium than a similar molecule with no superhelical turns (Pulleyblank and Morgan, 1975a; Burrington, 1977). Ethidium is a polycyclic aromatic compound possessing a planar conformation. These properties enable ethidium to intercalate itself between the bases in duplex DNA with a maximum frequency of one molecule per two base pairs (Waring, 1965). Binding of ethidium to duplex DNA enhances the fluorescence of ethidium by 25-fold (LePecq and Paoletti, 1967). Hence, when a negatively supercoiled DNA molecule relaxes, its ability to bind ethidium decreases which is reflected as a fluorescence decrease.

NC enzyme activity in extracts of rat prostatic nuclei produced a time dependent decrease in fluorescence enhancement as shown in Figure 6. The observed decrease was 28% after 20 min of incubation at 23°C. This is slightly below the value of 33% reported by Pulleyblank and Morgan (1975b) for the complete relaxation of PM2 DNA. However, in subsequent experiments, where the interval of incubation was extended to 60 min, the expected maximum of 33% was observed.

To confirm that the fluorescence assay was actually measuring a change in the superhelical state of PM2 DNA, the effect of an intact rat nuclear extract on the mobility of PM2 DNA in agarose gels was studied (Figure 7). After only 1 min of incubation, most of the fast running band of supercoiled PM2 DNA has been transfered to the slow moving position of the relaxed form. Native PM2 DNA is a more compact molecule than its relaxed counterpart (Vinograd and Lebowitz, 1966) which allows the native molecule to migrate faster through an agarose gel. This supports the contention that the observed change in fluorescence is due to relaxation of the supercoiled DNA substrate.

The agent able to relax PM2 DNA is a protein of unknown size and complexity (Chapter II.E.4.). The enzymatic character of the protein is supported by the following arguments. a. Diluted nuclear extracts that incompletely relax PM2 DNA are able to further relax the substrate if incubated for longer than 60 min. b. A nuclear extract only relaxes the negative supercoils of the PM2 DNA substrate; it does not introduce positive supercoils. However, a superhelix unwinding mechanism involving non-enzymatic protein binding might be expected to induce positive supercoiling. c. The sigmoidal dilution curve of the activity in the nuclear extracts (Chapter III.A.2.) is not typical of the

Figure 6 Kinetics of NC enzyme activity. An extract of intact rat prostatic nuclei was prepared and assayed as described in Chapter II.E. At various times after the start of the reaction, samples were removed and their fluorescence values measured.

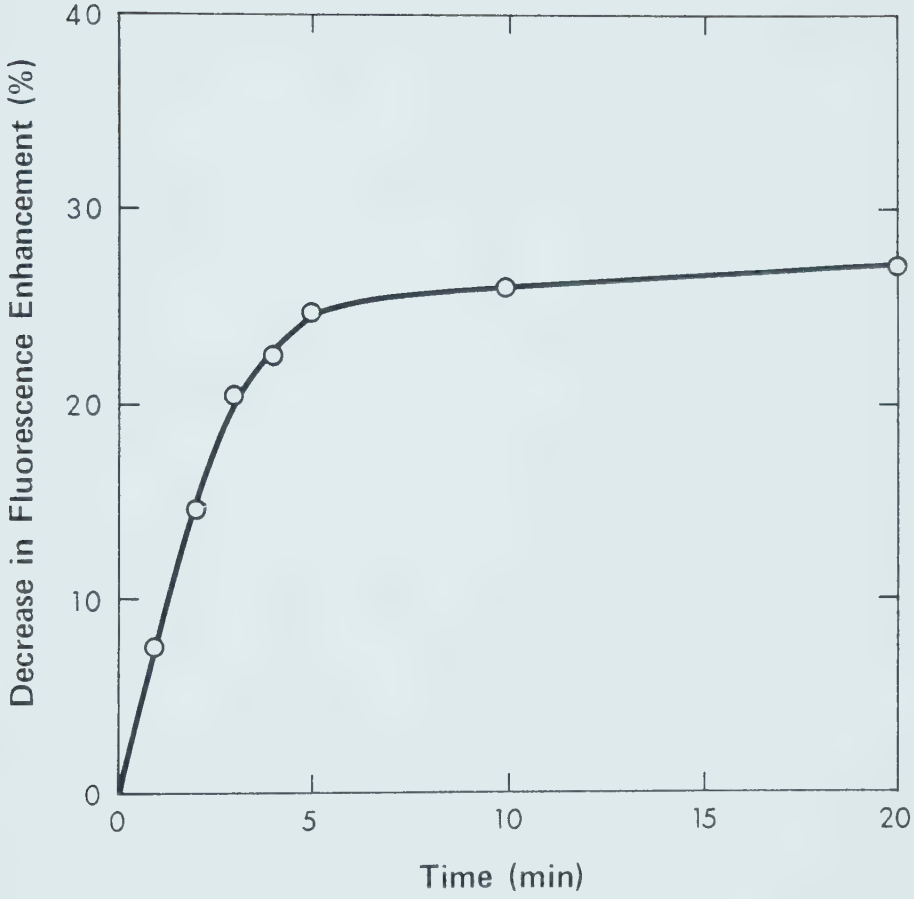
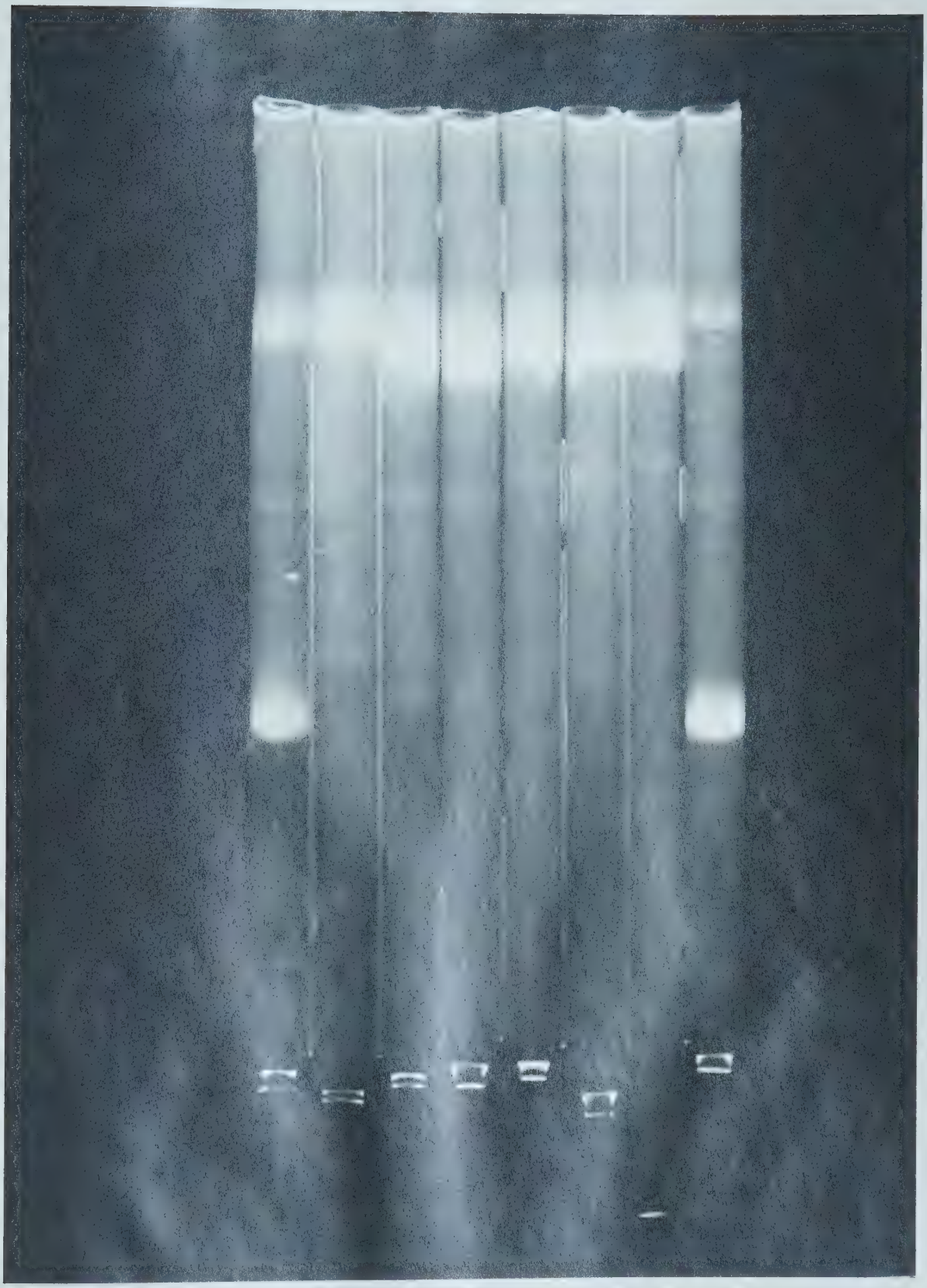

Decrease in Fluorescence Enhancement (%)
40
30
20
10
0
0
5
10
15
20
Time (min)

Figure 7 Agarose electrophoresis of PM2 DNA. An extract of intact rat prostatic nuclei was prepared and assayed as described in Chapter II.E. At various times after the start of the reaction, samples were withdrawn and the reaction stopped by repeated freezing in liquid nitrogen. Samples containing 1-2 μg PM2 DNA in a 25 μl vol (buffer D containing 10% glycerol) were loaded on 1.2% agarose cylindrical gels, electrophoresed and analyzed as described in Chapter II.F. Migration is from top to bottom, with the supercoiled PM2 DNA migrating most rapidly. Samples appear from left to right : zero time point taken immediately after addition of the nuclear extract; 1 min after addition of nuclear extract; 2 min; 3 min; 4 min; 5 min; 20 min; duplicate zero time point taken prior to addition of the nuclear extract.



kind expected if superhelix unwinding proceeds by non-enzymatic protein binding. Indeed, if the decrease in superhelix winding is caused by stoichiometric protein binding, one would expect a linear dilution curve. d. The nature of the fluorescence assay argues against any stoichiometric protein binding reaction causing superhelix unwinding. Placing the sample into a high pH buffer followed by heating at 100°C for 2 min are conditions that would destroy such protein-nucleic acid association. e. Addition of pronase to PM2 DNA just relaxed by a prostatic nuclear extract does not change the apparent degree of superhelix unwinding. All these observations can only be rationalized by the assumption that the protein responsible for superhelix unwinding is an enzyme.

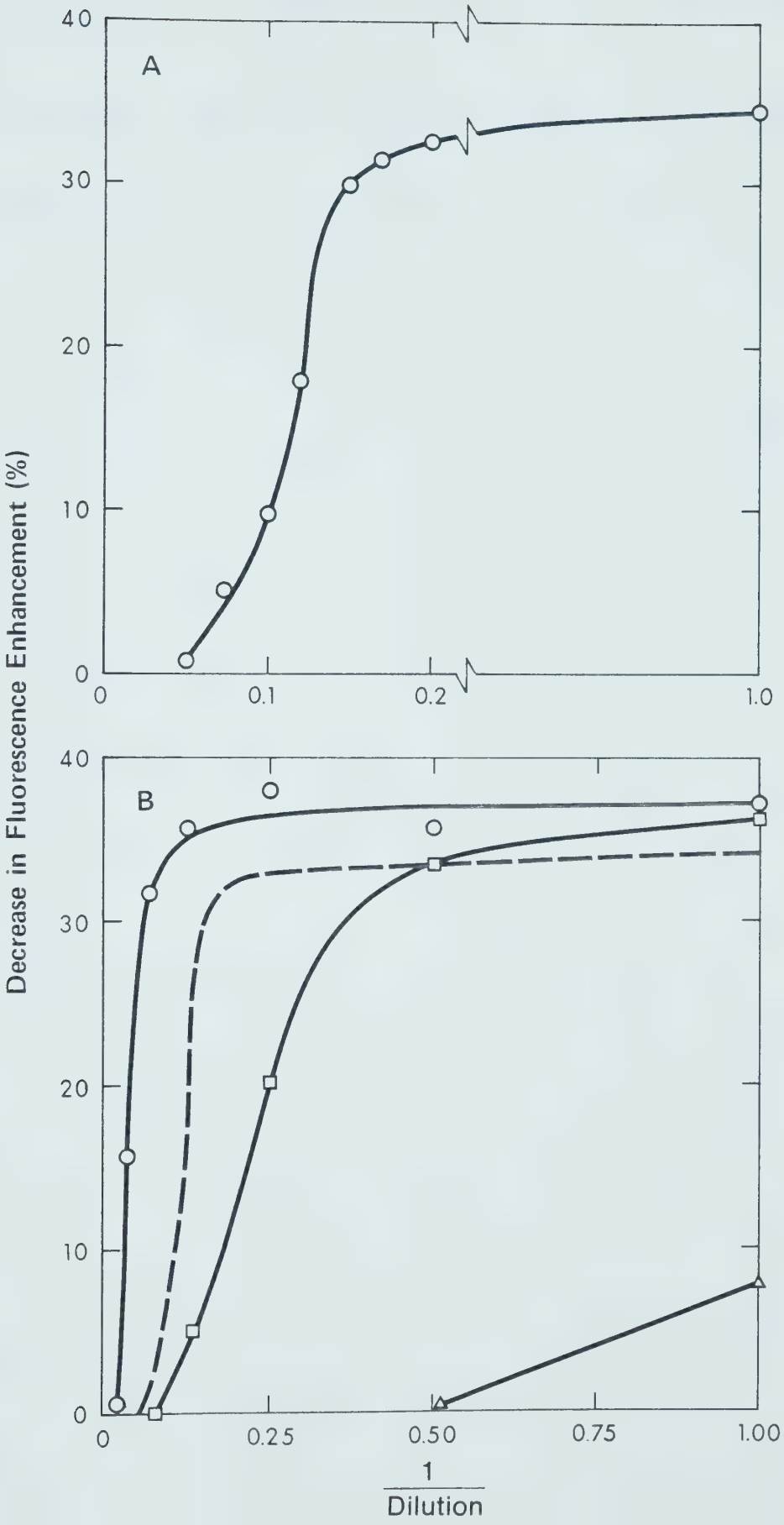
2. Quantitation of NC enzyme activity

It was necessary to determine the relative amounts of assayable NC enzyme activity present in various nuclear extracts. To obtain this information, a dilution curve was prepared from an extract of intact rat prostatic nuclei (Figure 8A). This sigmoidal curve was used to determine assayable levels of NC enzyme activity present in any nuclear extract, relative to that amount of activity measureable in a standard nuclear extract prepared from intact rat prostate. Figure 8B illustrates different types of dilution curves that can be obtained by assaying nuclear extracts containing different amounts of NC enzyme activity.

One unit was arbitrarily defined as that amount of activity present in an undiluted extract that when diluted 10-fold, will still produce 9.8% decrease in fluorescence enhancement in 60 min under the conditions of the fluorescence assay (Chapter II.E.3). The relative amounts of

Figure 8 A. Dilution curve of NC enzyme present in an extract of intact rat prostatic nuclei. The extract was prepared, diluted and each dilution was assayed as described in Chapter II.E. The sigmoidal nature of the dilution curve is made apparent by using an extended abscissa.

B. Comparison of various amounts of NC enzyme activity present in extracts of different nuclei. Extracts were prepared from nuclei isolated from different souces and assayed for NC enzyme activity as described in Chapter II.E. Each extract was prepared using a comparable volume of nuclei. Dilution curves for the following tissues, with their respective relative activities, are illustrated: (○) Noble rat prostatic carcinoma nuclei, 6.7 arbitrary unit; (□) Noble rat mammary carcinoma nuclei, 0.7 arbitrary units; (△) 7-day castrated rat prostatic nuclei, 0.1 arbitrary units. The dilution curve prepared from an extract of intact rat prostatic nuclei (Figure 8A) is shown as the dashed line.


A
B
40
30
20
10
0
0
0.1
0.2
1.0
0.25
0.50
0.75
1.00
Decrease in Fluorescence Enhancement (%)
1
Dilution

assayable NC enzyme activity present in the extracts is given by the following relation,

$$\text{arbitrary units} = \left(\frac{\%\ \text{decrease in FE of 1/n titre}}{9.8\%}\right) \times \left(\frac{n}{10}\right) \times \left(\frac{9.28 \times 10^4}{N}\right),$$

where FE represents the observed fluorescence enhancement (Chapter II.E.3), n is the titre of the extract dilution which produces an approximate 10% decrease in fluorescence enhancement, and N is the number of nuclei used to prepare the extract expressed per μl of extract. The first two ratios express the number of dilutions of a given nuclear extract required to observe an approximate 10% decrease in fluorescence enhancement relative to the 10-fold (n=10) dilution required with the intact rat prostatic nuclear extract, to produce a 9.8% decrease in fluorescence enhancement. The last term expresses the concentration of nuclei in the intact rat prostatic extracts, to that nuclear concentration present in any given extract. With this relation, it is possible to use the fluorescence assay for comparing quantities of assayable NC enzyme activity present in extracts of different nuclei.

Table 2 summarizes the relative amounts of assayable NC enzyme activity present in nuclei isolated from prostatic and mammary tissues. NC enzyme activity is present in human prostatic nuclei from normal, hyperplastic and carcinomatous tissue and in nuclei isolated from different transplantable tumours (Noble, 1977). The data suggests that some estrogen sensitive tumours may be characterized by unusually high NC enzyme activity. No appreciable differences in NC enzyme activity was observed between the various human tissues.

Table 2 Relative levels of assayable NC enzyme activity in various tissues. Nuclei were isolated using the high purity, low yield procedure. Nuclear extracts were prepared and levels of NC enzyme activity were determined as described in Chapter II. E. Human tissues were supplied by Dr. G. Lieskovsky. Rat tumours are transplantable neoplasms grown subcutaneously in Noble rats (Noble, 1977).

TABLE 2.

Relative Amounts of Assayable NC Enzyme Activity

in Prostatic and Mammary Tissues

Tissue	Description	NC enzyme activity (arbitrary units)
Human prostate	normal	2.1
	hyperplasia	2.0, 2.3
	carcinoma	2.1
Rat prostate	carcinoma, androgen stimulated	1.1
	carcinoma, estrogen stimulated	6.7
Rat mammary gland	carcinoma, estrogen dependent (line 1)	0.7
	carcinoma, estrogen dependent (line 2)	6.1

3. Hormonal dependence of NC enzyme activity

Androgens appear to influence the levels of assayable NC enzyme activity. Removal of androgens by castration results in a decrease of assayable NC enzyme activity present in rat ventral prostate nuclei (Figure 9). By 7-days after castration, assayable levels of activity in prostatic nuclei have dropped to 10% of normal. Administration of dihydrotestosterone immediately following castration, maintains normal levels of assayable NC enzyme activity in prostatic nuclei.

Figure 10 illustrates the restoration of NC enzyme activity levels in prostatic nuclei of 7-day castrated rats treated with daily subcutaneous injections of dihydrotestosterone. NC enzyme activity returns to normal levels by day 8 of the treatment and appears to be maintained with further treatment. The period of DNA synthesis, as determined by Lesser and Bruchovsky (1973), is presented to illustrate the time-sequence relationship between DNA synthesis and the return of NC enzyme activity. When DNA synthesis is maximal, NC enzyme activity levels are changing most rapidly. From day 2 to day 5, NC enzyme activity levels increase at a rate of 0.2 units per day. From day 5 to day 8 the rate of NC enzyme activity increase is less than 0.1 unit per day. Prostatic cell replication also increases most rapidly between day 2 and day 5 but after day 5, replication is markedly curtailed (Lesser and Bruchovsky, 1973).

The data contained in Table 3 demonstrates the inability of 3β-androstanediol, a physiologically inactive metabolite of dihydrotestosterone, to stimulate an increase in assayable NC enzyme activity comparable to that produced by dihydrotestosterone. Lesser (1974)

Figure 9 Androgen dependence of rat prostatic NC enzyme activity. Animals were castrated and killed at various times after castration. Prostatic nuclei were isolated from the disected ventral lobes, a nuclear extract was prepared and the level of assayable NC enzyme activity was determined as described in Chapter II.E. The variation of NC enzyme activity in prostates of rats not receiving dihydrotestosterone is represented by circles (○). NC enzyme activity levels in prostates of rats receiving daily subcutaneous injections of dihydrotestosterone (400 µg per 100 g body weight) are represented by squares (□). Each data point expresses the result obtained from a single experiment.

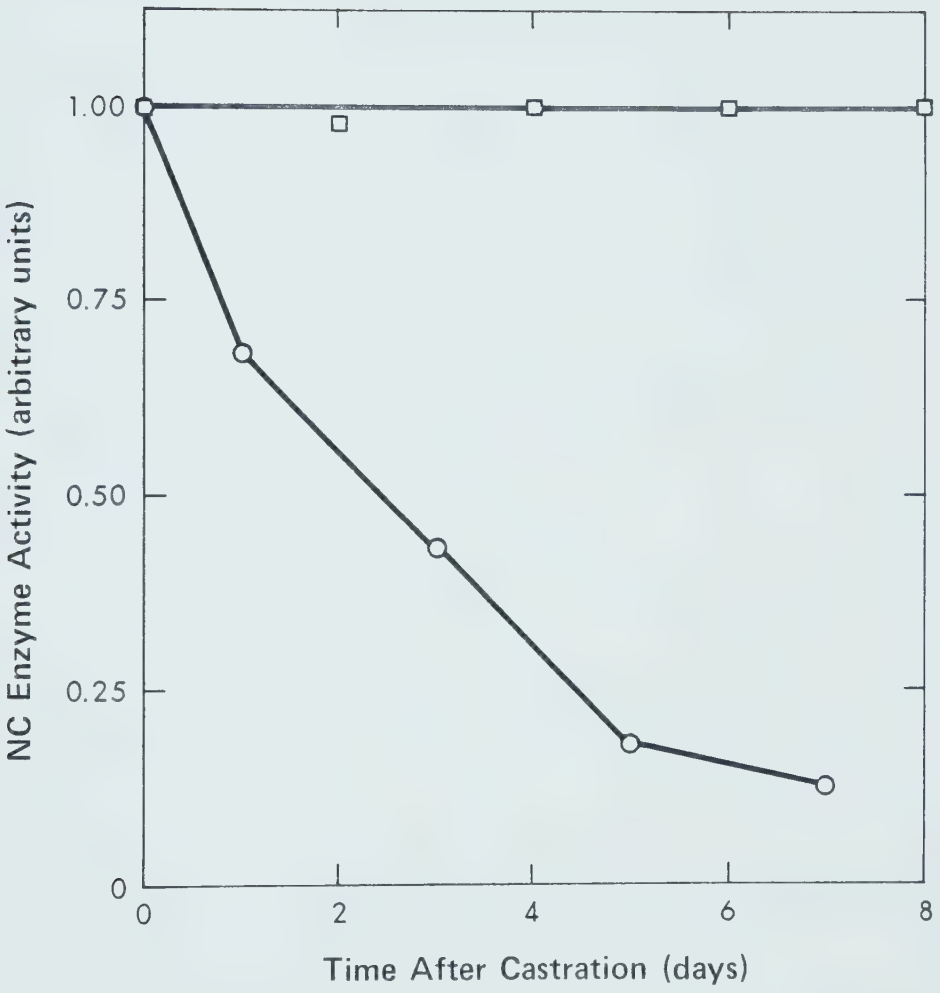

1.00
0.75
0.50
0.25
0
0
2
4
6
8
NC Enzyme Activity (arbitrary units)
Time After Castration (days)

Figure 10 Restoration of NC enzyme activity in prostatic nuclei of 7-day castrated rats receiving hormone replacement therapy. Beginning at 7 days post-castration, rats received daily subcutaneous injections of 400 μg of dihydrotestosterone per 100 g body weight. At various times after initiation of hormone replacement therapy, the rats were killed, ventral prostate lobes were disected and the nuclei were isolated. Extracts of isolated nuclei were prepared and the level of assayable NC enzyme activity was determined as described in Chapter II.E. The results are expressed as the mean of two independent experiments. The dashed line illustrates the variation in DNA synthesis under similar conditions (Lesser and Bruchovsky, 1973).

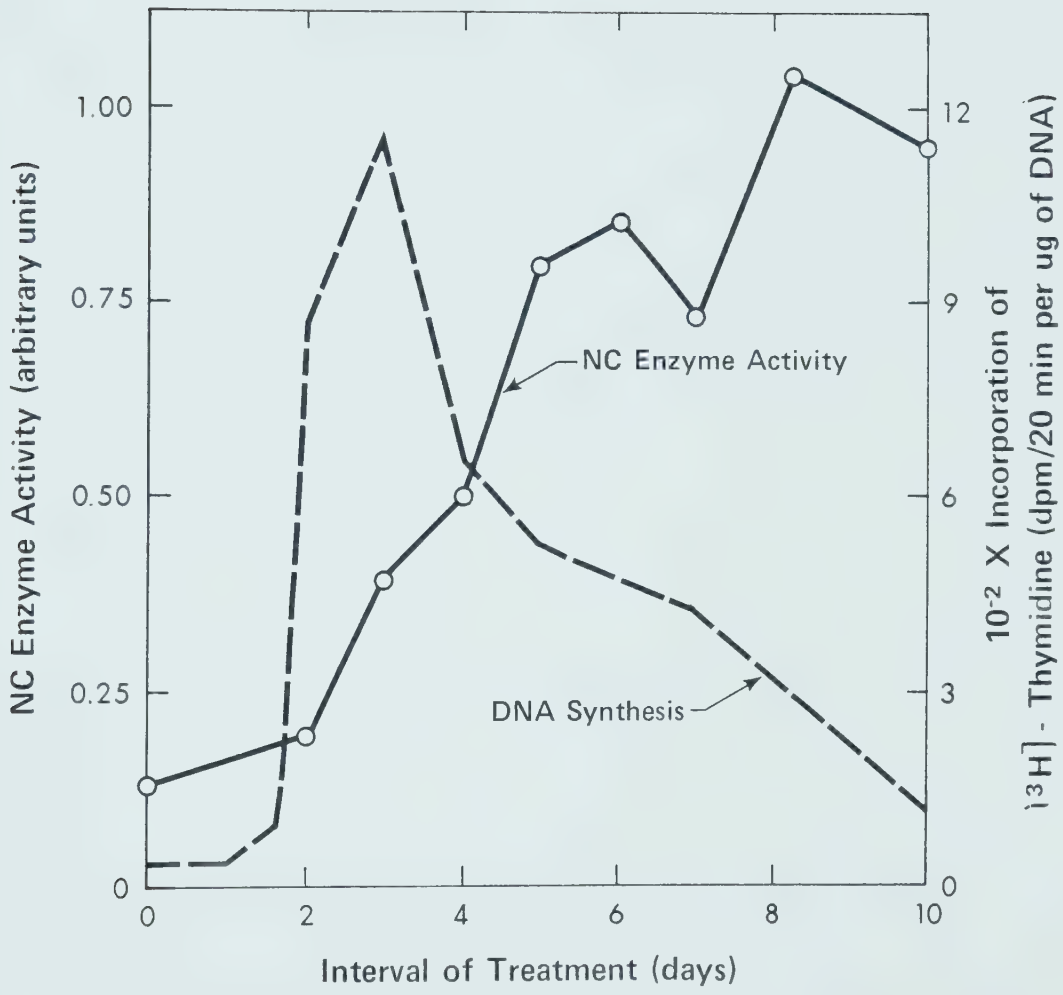

NC Enzyme Activity (arbitrary units)
1.00
0.75
0.50
0.25
0
0
2
4
6
8
10
Interval of Treatment (days)
12
9
6
3
0
10-2 X Incorporation of [3H]- Thymidine (dpm/20 min per ug of DNA)
NC Enzyme Activity
DNA Synthesis

Table 3 The effect of 3β-androstanediol on prostatic NC enzyme activity of 7-day castrated rats. Beginning 7 days after castration, animals received daily subcutaneous injections of 400 μg 3β-androstanediol per 100 g body weight. At various times after the initiation of treatment, the animals were killed, the ventral prostate lobes removed and extracts were prepared from the isolated nuclei. The level of NC enzyme present in the extracts was then determined. The data contained in the table are the results of single experimental determinations.

TABLE 3.

Absence of an Effect of 3β-Androstanediol on Assayable NC Enzyme Activity

Interval of treatment with 3β-androstanediol (days)	NC enzyme activity (arbitrary units)
untreated control	0.1
1	0.1
2	0.1
3	0.1
4	0.2

also found that 3β-androstanediol was unable to stimulate levels of DNA synthesis comparable to that of dihydrotestosterone. These results strongly suggest that assayable levels of NC enzyme activity in prostatic nuclei are sensitive to fluctuations in the circulating level of active androgen only.

B. Structure of Rat Prostatic Chromatin

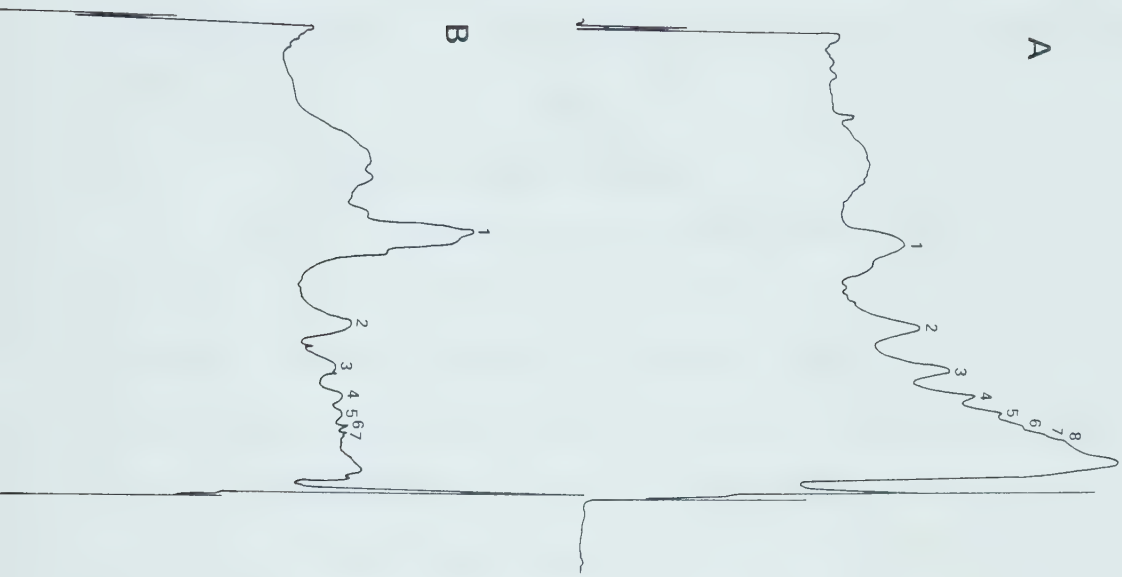
The structure of rat prostatic chromatin was studied using micrococcal nuclease to preferentially hydrolyze internucleosomal DNA, producing DNA fragments of various size. Size analysis of these fragments yielded information concerning the nucleosome repeat length, the nucleosome core size and the arrangement of nucleosomes along the chromatin fibre. Structure analysis of chromatin from intact rat prostate and 7-day castrated rat prostate was performed to study the effect of the removal of androgens on the structure of rat prostatic chromatin.

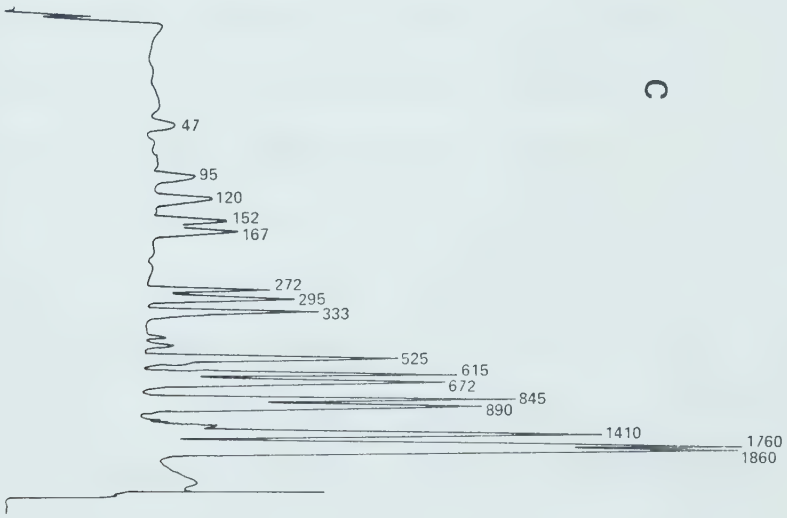
1. Repeating nature of micrococcal nuclease generated DNA fragments.

Limited micrococcal nuclease digestion of prostatic nuclei isolated from intact and 7-day castrated rats generates DNA fragments which are multiples of a basic repeating unit. Figure 11 contains the absorbance tracings prepared from gels used to separate such DNA fragments. Fragments representing DNA contained in the repeating unit (monomer) and multiples of up to 6 and 7 repeating units (oligomers) can be resolved. The fastest running material is most likely DNA fragments produced from nuclease digestion of core DNA, along with small oligonucleotides produced from nuclease digestion of internucleosomal DNA (spacer regions).

Figure 11 Absorbance profiles of slab gels used to analyze DNA fragment size. Nuclei from prostates of intact and 7-day castrated rats were isolated and incubated with micrococcal nuclease. The isolated DNA fragments were electrophoresed on 3% acrylamide : 0.5% agarose composite slab gels. The gels were stained with Stains-all and scanned at 600 nm using a spectrophotometer equipped with a gel scanning accessory. Direction of migration is from right to left.

A. DNA fragments isolated from intact rat prostatic nuclei previously incubated with micrococcal nuclease (1.8% digestion).
B. DNA fragments isolated from 7-day castrated rat prostatic nuclei previously incubated with micrococcal nuclease (3.1% digestion). Arabic numerals 1,2,3 etc. denote DNA fragments isolated from monomers, dimers, trimers etc.
C. Hae III restriction endonuclease fragments of PM2 DNA. Molecular sizes (base pairs) are taken from Kovacic and Van Holde (1977).


B
1
2
3
4
5
6
7
A
1
2
3
4
5
6
7
8


C
47
95
120
152
167
272
295
333
525
615
672
845
890
1410
1760
1860

These DNA fragments were sized by co-electrophoresing Hae III restriction endonuclease fragments of PM2 DNA. A tracing of these fragments as they appear on a scan of the electrophoresis gel is shown in Figure 11C. Gels were calibrated by plotting the logarithmic size of each PM2 DNA fragment against its migrated distance (Figure 12). DNA fragments ranging in length from 47 base pairs to approximately 1800 base pairs could be sized.

2. Nucleosome repeat length

Average nucleosome repeat lengths were obtained by subtracting the size of each oligomer from that of the next higher oligomer (hexamer - pentamer, pentamer - tetramer, tetramer - trimer, etc.). These values were then averaged to yield a single determination of the average nucleosome repeat length. This method of determining the average nucleosome repeat length has the advantage of compensating for nuclease degradation at the ends of the oligomers (Morris, 1976a; Noll and Kornberg, 1977). Repeat lengths determined by dividing the DNA fragment size by the oligomer number (monomer ÷ 1, dimer ÷ 2, trimer ÷ 3, etc) are likely to be in error since micrococcal nuclease can hydrolyze the ends of oligomers to produce DNA fragments whose size is considerably smaller than if the nuclease did not further process oligomers. This problem is important when analyzing the small oligomers and oligomers arising from prolonged nuclease digestion.

As shown in Table 4, the average repeat lengths obtained for intact and 7-day castrated rat prostatic chromatin at 218 and 223 base pairs respectively, do not differ significantly. This point is further illustrated in Figure 13, where the size of each oligomer is plotted against its oligomer number. Such a plot yields a straight line with a

Figure 12 A standard curve used to calibrate 3% polyacrylamide : 0.5% agarose composite slab gels. Each slab gel had one lane containing Hae III restriction endonuclease fragments of PM2 DNA for the purpose of molecular size calibration.

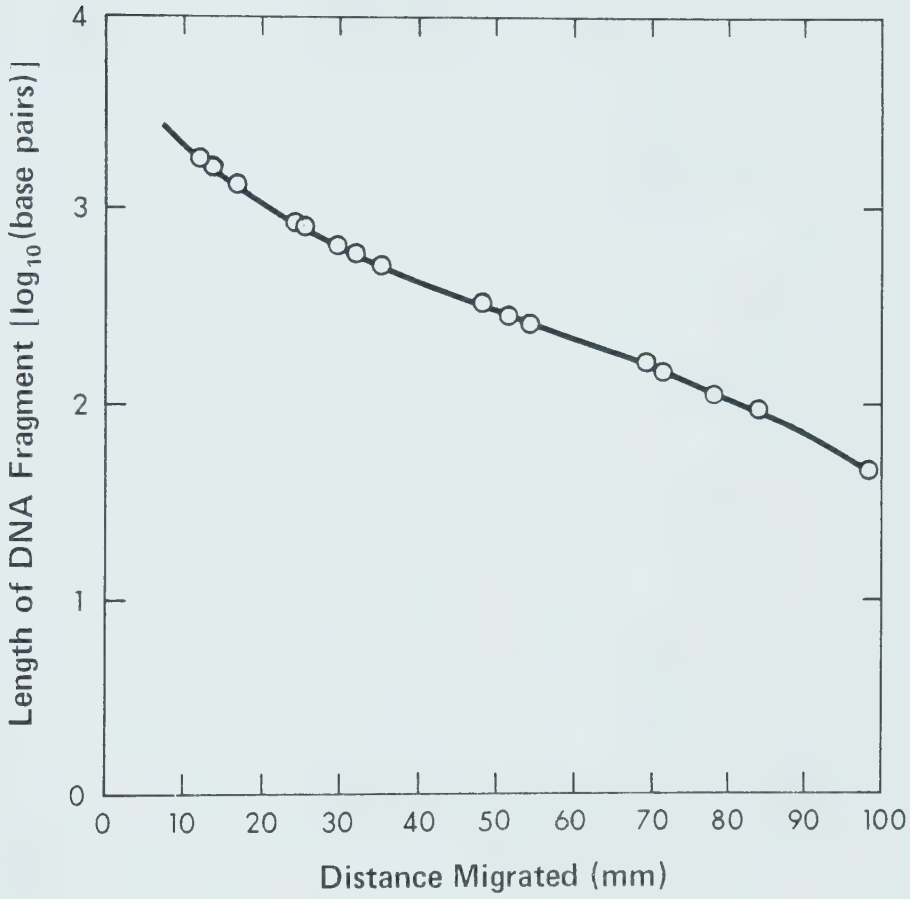

Length of DNA Fragment [log10(base pairs)]
4
3
2
1
0
0
10
20
30
40
50
60
70
80
90
100
Distance Migrated (mm)

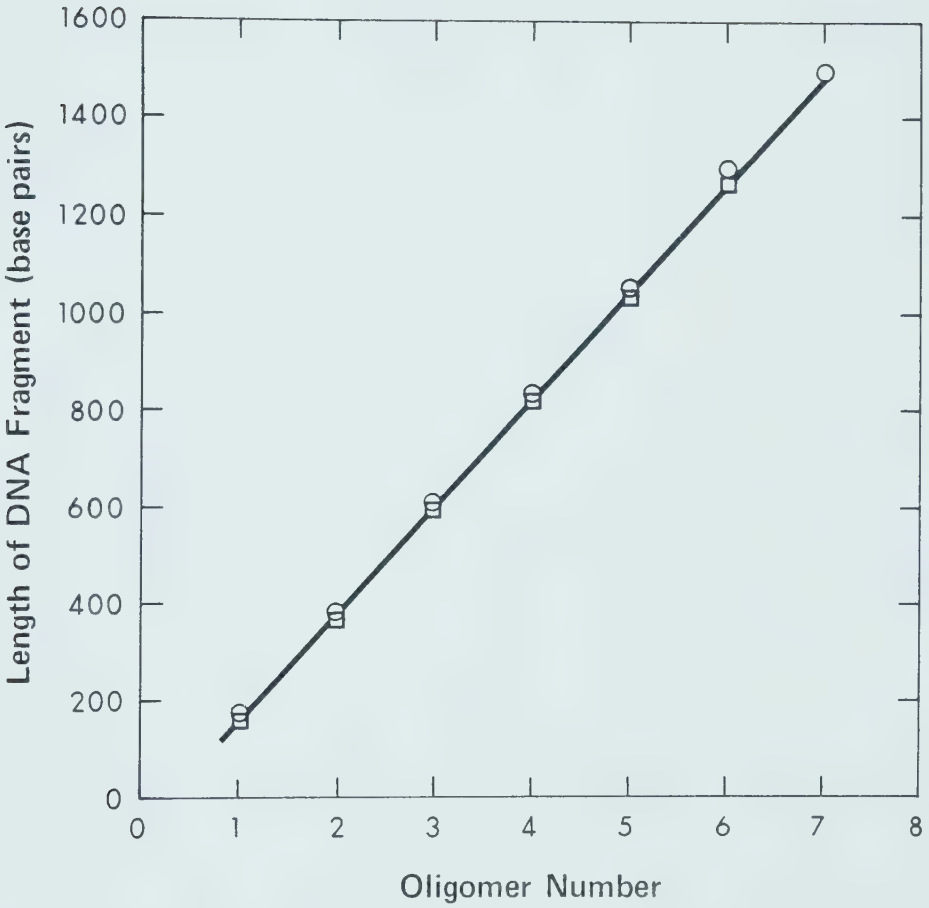
Table 4 Determination of the average nucleosome repeat length. Nuclei from prostates of intact and 7-day castrated rats were isolated and incubated with micrococcal nuclease. Micrococcal nuclease generated DNA fragments were isolated and electrophoretically analyzed on 3% acrylamide : 0.5% agarose composite slab gels as described in Chapter II.K. Nucleosome repeat lengths obtained for each % digestion were calculated by subtracting the size of each oligomer from that of the next higher oligomer and averaging the values (Chapter III.B.2). Values shown in the table represent the average ± S.D. from 2-10 independent electrophoretic experiments.

TABLE 4.

Average nucleosome Repeat Length in Chromatin from Prostates of Intact and 7-day Castrated Rats

Intact rat prostatic chromatin		7-Day castrated rat prostatic chromatin	
% Digestion	Repeat length (base pairs)	% Digestion	Repeat length (base pairs)
1.4	213 ± 6 (3)	1.0	221 ± 8 (8)
1.8	223 ± 11 (10)	1.8	225 ± 6 (7)
2.0	222 ± 4 (3)	2.5	227 ± 3 (3)
3.6	217 ± 12 (10)	3.1	229 (2)
5.3	217 ± 6 (4)	4.7	222 ± 9 (3)
6.0	213 ± 8 (4)		
7.6	214 ± 7 (3)		
8.2	221 ± 2 (4)		
overall mean	218 ± 9 (41)	overall mean	223 ± 6 (23)

Figure 13 Comparison of the nucleosome repeat length of prostatic chromatin from intact and 7-day castrated rats. Nuclei were isolated and incubated with micrococcal nuclease (1.8% digestion). The length of micrococcal nuclease generated DNA fragments are plotted against the oligomer number, n (for a monomer n = 1, dimer n = 2, trimer n = 3 etc.). Each data point in the average of between 2 and 10 separate determinations. Results from 7-day castrated rat prostatic chromatin are represented by circles (○) and those from intact rat prostatic chromatin, by squares (□).


Length of DNA Fragment (base pairs)
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
Oligomer Number

slope equal to the average nucleosome repeat length (Morris, 1976b).

Thus, micrococcal nuclease digestion of intact and 7-day castrated rat prostatic nuclei produced DNA fragments that reflect the structure of chromatin from these two sources. These results agree with the currently accepted theory of chromatin structure (Felsenfeld, 1978), where DNA is regularly arranged into nuclease sensitive regions (spacer regions) and nuclease resistant regions (nucleosome cores). The average nucleosome repeat lengths for both types of prostatic chromatin are the same and these values are within the range of nucleosome repeat lengths found in other eukaryotic chromatin (Felsenfeld, 1978).

3. Nucleosome core size and structure

The internal structure of nucleosome cores was estimated from the acrylamide:agarose composite gels by analyzing the fast moving DNA fragments. When clearly identifiable peaks could be measured from the material running faster than monosomes, consistent sizes for these fragments were obtained. These fragments are, therefore, thought to arise from digestion of the nuclease-resistant core DNA. The size distribution of these fragments is an indication of the accessibility of micrococcal nuclease to nucleosome core DNA and can therefore be regarded as a reflection of the core structure of nucleosomes. Values for core sizes were obtained by measuring the length of the DNA fragment in the fast migrating shoulder which often appears adjacent to the monomer peak.

The pattern of core DNA fragments thus obtained from nuclease digestion of intact and 7-day castrated rat prostatic chromatin are virtually identical (Table 5). The only difference is a 74 base pair

Table 5 Sizes of the fast running DNA fragments. The sizes of identifiable peaks were measured from the material migrating faster than monosomes on 3% acrylamide : 0.5% agarose composite slab gels. Values contained in the table are the mean ± S.D. of the number of independent determinations (shown in parentheses).

TABLE 5.

Size of Fast Running DNA Fragments

Intact rats	7-Day castrated rats
Repeat length (base pairs)	Repeat length (base pairs)
139 ± 7 (11)	136 ± 4 (4)
114 ± 4 (6)	117 (1)
107 ± 2 (5)	105 ± 1 (4)
89 ± 7 (6)	94 ± 1 (4)
66 ± 1 (4)	74 ± 1 (4)
59 ± 1 (4)	64 ± 3 (4)
47 ± 1 (3)	54 ± 2 (4)
	49 (1)

fragment observed in the 7-day castrated rat prostatic nuclear digestions. The size of the DNA fragment contained in the nucleosome core and the pattern of DNA fragments presumed to arise from intranucleosomal core digestion are comparable to the results of others using micrococcal nuclease to study the organization of eukaryotic DNA into nucleosomes (Compton et al, 1976; Lohr et al, 1977; Whitlock, 1977).

4. Organization of nucleosomes

The relation between oligomer size and % digestion can be used to infer whether nucleosomes are arranged regularly or irregularly along the chromatin fibre (Lohr et al, 1977). If nucleosomes are spaced irregularly on the chromatin fibre, then as nuclease digestion proceeds, the oligomers possessing widely spaced nucleosomes would be hydrolyzed to smaller oligomers faster than those same oligomers possessing closely spaced nucleosomes. Therefore, the size of any given oligomer population would appear to decrease as a function of % digestion. In contrast, regularly spaced nucleosomes would yield oligomers whose size remained constant over a relatively large range of digestion.

Digestion profiles of chromatin from intact and 7-day castrated rat prostates appear in Figure 14. The data indicates that for either chromatin, the size of any given oligomer does not change with increasing % digestion. As shown in Table 6, observed oligomer length is only slightly greater than the value expected if nucleosomes were arranged in an orderly, regular manner.

These digestion profiles therefore, suggest that the nucleosomes

Figure 14 Prostatic chromatin digestion profiles. Nuclei isolated from the prostates of intact and 7-day castrated rats were incubated with micrococcal nuclease. DNA was isolated and electrophoretically analyzed on 3% acrylamide : 0.5% agarose composite slab gels. Each line represents the length of a given oligomer as a function of increasing digestion by micrococcal nuclease. The size of oligomers at zero % digestion was calculated by multiplying the nucleosome repeat length by the appropriate oligomer number.

A. DNA fragments isolated from intact rat prostatic nuclei previously incubated with micrococcal nuclease.
B. DNA fragments isolated from 7-day castrated rat prostatic nuclei incubated with micrococcal nuclease. Results are expressed as the mean of between 2 and 10 separate determinations.

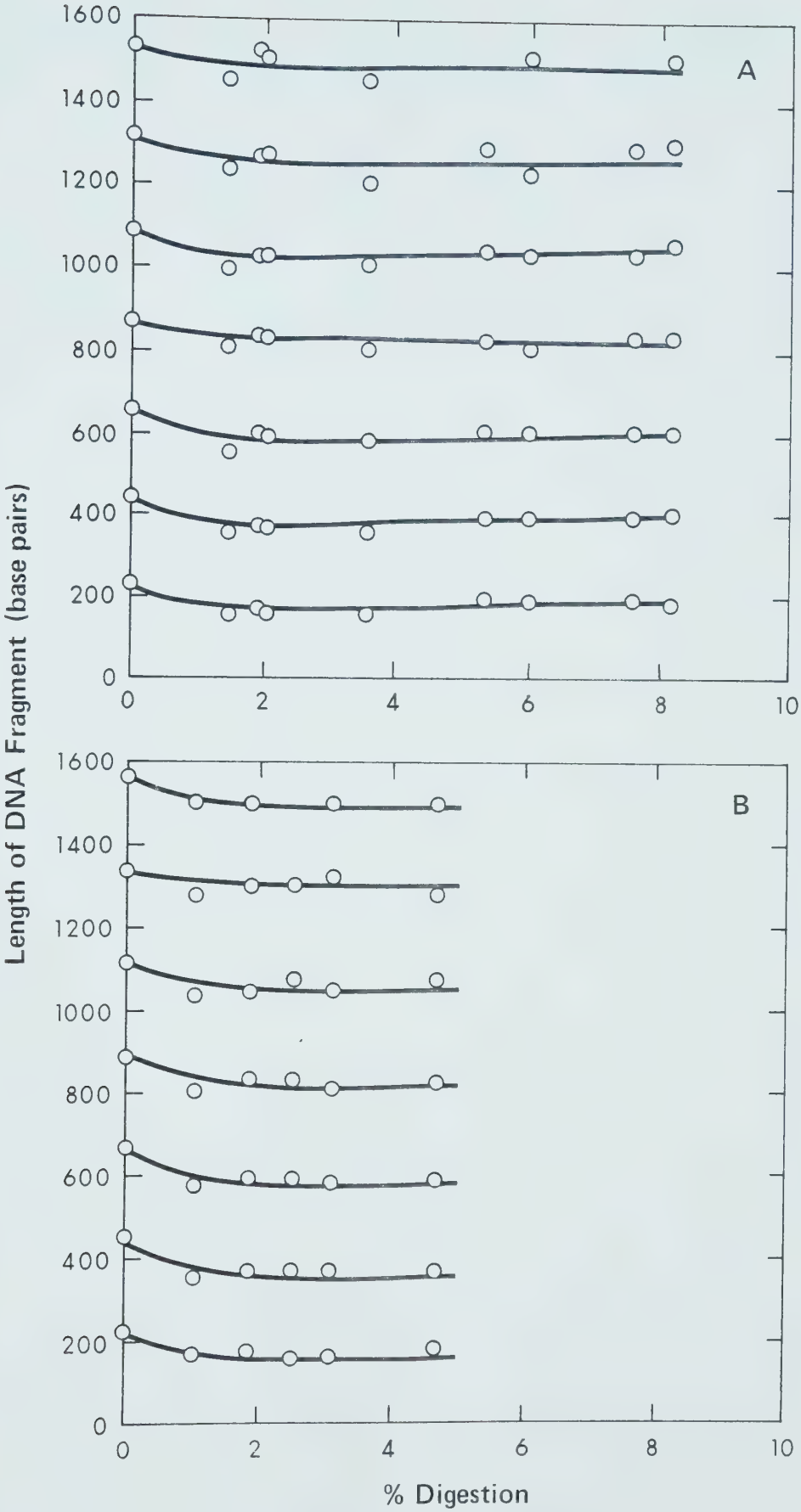

A
B
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
Length of DNA Fragment (base pairs)
% Digestion

Table 6 Comparison of expected and observed lengths of oligomers produced by the micrococcal nuclease digestion of intact and 7-day castrated rat prostatic nuclei. The following relation was used to calculate the expected values:

(repeat length x oligomer number) - (spacer length)

based upon the assumption that the arrangement of nucleosomes along the chromatin fibre is regular. Repeat lengths used in the calculation are from Table 4 and the spacer length was determined by subtracting the nucleosome core size (Table 5) from the repeat length. Observed oligomer lengths are taken from Figure 14.

TABLE 6.

Comparison of Expected and Observed Lengths of Oligomers Produced by the Micrococcal Nuclease Digestion of Intact and Castrated Rat Prostatic Nuclei

	Intact rat prostatic chromatin		7-day castrated rat prostatic chromatin	
Oligomer number (n)	Expected (218n - 79)	Observed	Expected (223n - 87)	Observed
7	1447	1490	1474	1500
6	1229	1270	1251	1300
5	1011	1030	1028	1040
4	793	820	805	820
3	575	600	582	590
2	357	380	359	370
1	139	180	136	170

are arranged in a regular manner along the fibre of rat prostatic chromatin. The gross structural organization of chromatin does not change in the 7-day castrated rat prostate.

CHAPTER IV

DISCUSSION

The purpose of this work was to gain an understanding of the events underlying steroid hormone action by studying the effect of androgens on the biochemistry of the prostate gland. Two biochemical aspects of the prostate gland were studied, one functional and one structural. The activity of an enzyme thought to be involved in such processes as DNA replication and transcription was assayed as a function of hormone stimulation. It was hoped that the presence of this enzyme could be correlated with cellular proliferation and differentiation. Also, the structure of chromatin from intact and castrated rat prostates was compared to determine whether any structural differences could be related to the biochemical and functional differences that distinguish the normal and involuted prostate glands. The differentiated state of normal prostatic cells is probably characterized by a unique pattern of transcriptional specificity and frequency, quite different from that of involuted prostatic cells (Parker and Mainwaring, 1977). Since the structure of chromatin is a possible transcriptional control element, any differences in the frequency and specificity of transcription may be reflected by chromatin structure.

NC enzyme is though to be necessary for DNA replication and may also be involved in the regulation of DNA transcription (Wang, 1973; Burrington, 1977). Current ideas concerning the regulation of DNA replication and transcription focus upon the possibility that superhelical coiling of DNA may be an element of control. NC enzyme is

proposed as the agent responsible for altering supercoiling of DNA templates.

Most models of DNA replication predict the formation of superhelical DNA ahead of and/or behind the replication fork, as a consequence of DNA replication (Champoux and Delbecco, 1972; Wang, 1973; Burrington and Morgan, 1976). Since the introduction of supercoils into duplex DNA occurs with an increase in free energy, some agent must be present to relieve this topological constraint or DNA replication would be inhibited soon after its initiation. NC enzyme has been proposed to perform this task (Champoux and Delbecco, 1972; Wang, 1973; Burrington and Morgan, 1976).

Frequency and specificity of DNA transcription may also be affected by superhelical coiling of DNA templates. DNA templates that have negative superhelical twists appear to behave as better templates for transcription than relaxed or positively supercoiled DNA molecules (Botcham et al, 1973; Richardson, 1974).

The work contained in this thesis demonstrates the presence of an enzyme in rat prostatic nuclei, capable of relaxing negatively supercoiled DNA. The ability of this enzyme to relax positive supercoils was not determined, although other eukaryotic NC enzymes are able to relax both positive and negative supercoils (Champoux and Delbecco, 1972; Champoux and MacConaughy, 1975; Keller, 1975; Pulleyblank and Morgan, 1975b; Vosberg et al, 1975; Mattoccia, et al, 1976; Vosberg and Vinograd, 1976). The NC enzyme from rat prostate was studied only as a crude nuclear extract and other characteristics of the protein were not investigated.

A method for comparing relative amounts of NC enzyme activity present in extracts of different nuclei was developed. Variations in nuclear NC enzyme activity could then be quantitatively related. Prostatic NC enzyme activity levels appear to be influenced by hormones. Castration causes a 90% reduction in NC enzyme activity levels after 7 days. Normal activity levels in castrated rat prostatic nuclei can be maintained by daily injections of dihydrotestosterone. Furthermore, normal activity levels can be restored in 7-day castrated rat prostatic nuclei by 8 days of dihydrotestosterone treatment. NC enzyme activity variation cannot be explained by changes in the proportion of epithelial nuclei that are isolated from prostatic tissue during the various stages of involution and regrowth. The use of a tissue-mincing stainless steel screen during the nuclear isolation procedure effectively separates epithelial cells from stromal cells. Hence, the same type of nucleus is assayed and differences in NC enzyme activity are not attributable to changing proportions of epithelial nuclei used to prepare extracts.

It was thought that the function of prostatic NC enzyme might be studied if the hormone-induced variation of enzyme activity was examined relative to changes in cellular proliferation and differentiation that occur in the involuting and regenerating rat prostate glands. The observation that the rate of increase of NC enzyme activity in regenerating rat prostate is greatest during the period of DNA synthesis suggests the possibility that the process of DNA synthesis and the function of prostatic NC enzyme are related. However, assayable levels of NC enzyme activity still increase after DNA synthesis and cellular proliferation have abated. This implies

that NC enzyme may have functions other than with respect to DNA synthesis.

The rat prostate gland is a nonproliferating, differentiated tissue requiring only enough DNA synthesis to maintain a low rate of cell turnover (Lesser, 1974). The involuted prostate of a 7-day castrated rat also has a low rate of DNA synthesis in the absence of androgen supplementation. Only sufficient DNA synthesis is required to maintain the size of the involuted tissue (Lesser, 1974). If the rates of DNA synthesis and cell turnover in these two otherwise different tissues are comparable, then there must be a reason for the observed 10-fold difference in NC enzyme activity. A possible explanation might lie in the most obvious difference between the two tissues, that is, the normal prostate is differentiated while the involuted gland is not. It is interesting to speculate that the higher amount of NC enzyme activity reflects the differentiated state of the normal tissue. This would then imply that NC enzyme may also be involved in regulation of transcription, since both the frequency and specificity of transcription are certain to be different in these two tissues (Parker and Mainwaring, 1977).

The mechanism by which hormone-induced changes in prostatic NC enzyme activity occur is not known. Changing levels of enzyme activity do not necessarily reflect differences in enzyme concentration. A number of mechanisms involving changing levels of inhibitor, coenzymes or activators could as easily explain the observed variations of enzyme activity.

The second part of this project concerned the influence of hormones on the structure of prostatic chromatin. Micrococcal

nuclease was used to study the nucleosome structure of chromatin from intact and 7-day castrated rat prostates. As previously mentioned, if the prostate is deprived of androgens, it involutes to a structurally and functionally undifferentiated tissue. The objective was to determine whether any differences could be detected in the structure of chromatin from normal and involuted tissues. The characteristics of chromatin structure studied were the average nucleosome repeat length, the internal structure of the nucleosome core and the arrangement of nucleosomes along the chromatin fibre.

The average nucleosome repeat length of prostatic chromatin is the same in the intact and the 7-day castrated rat. These repeat lengths are comparable to values reported for other sources (Felsenfeld, 1978).

The nucleosome core structure of intact and 7-day castrated rat prostatic chromatin are very similar, as suggested by the comparable sizes of fast running DNA fragments. The lengths of nucleosome core fragments produced by micrococcal nuclease digestion of rat prostatic chromatin are also similar to lengths of core fragments produced in micrococcal nuclease digestion of chromatin from other eukaryotic sources (Compton et al, 1976; Lohr et al, 1977; Whitlock, 1977). Unequivocal proof that these DNA fragments actually resulted from nuclease digestion of core DNA was not obtained. The origin of these fragments might be confirmed by showing that extended nuclease digestion of prostatic chromatin and digestion of individual monomers also give rise to DNA fragments of similar size.

The arrangement of nucleosomes along the prostatic chromatin fibre does not change following castration. Chromatin from intact and

7-day castrated rat prostates each possess a regular arrangement of nucleosomes. Lohr et al (1977) suggest that nucleosome arrangement might reflect the transcriptional activity of the chromatin. They postulate that actively transcribed chromatin possesses an irregular arrangement of nucleosomes, while inactively transcribed chromatin has a regular arrangement. Since it is unlikely that prostatic chromatin from 7-day castrated rats is transcribed with the same frequency and specificity that is characteristic of prostatic chromatin from intact rats (Parker and Mainwaring, 1977), the hypothesis of Lohr et al (1977) appears not to be supported by this data.

The structural similarity of chromatin from intact and 7-day castrated rat prostates agrees with recently published work on the structure-function relationship of chromatin. Experiments using micrococcal nuclease to probe the structure of chromatin have found that nucleosomes appear to be associated with both actively and inactively transcribed chromatin and that nucleosomes are not associated with any specific sequences of DNA (Axel et al, 1975; Lacy and Axel, 1975; Mathis and Gorovsky, 1976; Reeves and Jones, 1976; Garel et al, 1977; Reeves, 1977; Gotesfeld and Melton, 1978). Micrococcal nuclease does not seem to be able to distinguish between the nucleosome structure of actively transcribed and quiescent chromatin.

It appears that there are structural differences between transcriptionally active and quiescent chromatin (Foe et al, 1976; Garel and Axel, 1976; Scheer et al, 1976; Weintraub and Groudine, 1976; Woodcock et al, 1976; Zentgraf et al, 1976). These differences may be an absence of nucleosomes as in ribosomal genes or it may be an altered

nucleosome conformation as in non-ribosomal genes. DNase I but not micrococcal nuclease is able to detect the differences between non-ribosomal genes that are transcribed at diverse rates. Micrococcal nuclease can yield information regarding nucleosome repeat lengths, nucleosome core structure and arrangement of nucleosomes along the chromatin fibre. However, this information usually results from digestion of bulk chromatin, so that the selectivity needed to analyze specific genes is lacking. It may well be that certain genes in prostatic chromatin alter their characteristic nuclease core structure, repeat length or arrangement, upon hormonal deprivation. Unfortunately such changes would go unnoticed against the background properties of the bulk chromatin.

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